Unraveling Mechanisms of Transcriptional Repression: Novel Insights from Brinker

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UNRAVELING MECHANISMS OF TRANSCRIPTIONAL REPRESSION: NOVEL INSIGHTS FROM BRINKER

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Transcriptional repressors bind *cis*-regulatory elements of target genes in a sequence specific manner. To antagonize transcription, repressors primarily function by recruiting accessory proteins, co-repressors, which in turn largely function by modifying chromatin structure. Although a repressor could function by recruiting just a single co-repressor, many recruit more than one, with Brinker (Brk) from *Drosophila* recruiting the co-repressors, CtBP and Groucho (Gro), in addition to possessing a third repression domain, 3R. Previous studies indicated that Gro is sufficient for Brk to repress target genes in the wing imaginal disc, questioning why it should need to recruit CtBP, a 'short-range' co-repressor compared to Gro that can function over longer distances. To resolve this I have used genomic engineering to generate a series of endogenous *brk* mutants that are unable to recruit Gro, CtBP and/or have the 3R domain deleted. Analysis of these mutants reveals that while the recruitment of Gro is necessary and is almost sufficient for Brk to make a morphologically wild-type fly, it is insufficient during oogenesis where Brk must utilize CtBP and 3R to pattern the egg-shell appropriately. Gro insufficiency during oogenesis can be explained by its downregulation in Brk-expressing cells through phosphorylation downstream of EGFR signaling, thus making it unavailable for Brk which must then resort to CtBP and/or 3R for repressive activity. The present study dissects the mechanism of activity of a transcription factor and its co-repressors and is the first to do so in multicellular

eukaryotes in a physiologically relevant manner; additionally its findings provide a better understanding of why transcription factors in general may utilize more than one co-repressor.

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PREFACE

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1.0 REGULATION OF EUKARYOTIC GENE EXPRESSION

1.1 INTRODUCTION

The process by which the unicellular fertilized egg develops into a multicellular organism is one of the most profound problems in all of biology. How do multitudes of cells that arise from a single cell differentiate into distinct cell-types and become organized into a diversity of tissues and organs making up a whole organism? As both the zygote and the cells derived from it contain the same genomic information, a fundamental problem emerges: How does an identical static code get interpreted to generate the enormous variety of precise spatiotemporal gene expression patterns at each developmental stage and in every cell?

Differential gene expression is crucial for development, pattern formation and maintenance of homeostasis; it involves cells responding to distinct spatial and temporal cues from their microenvironment, and as a result a subset of their genes are turned on or off and this ultimately controls their identity and function. The cues that cells respond to are primarily secreted signaling polypeptides that act via cell membrane receptors and intracellular signaling machinery to modulate the activity of transcription factors eventually leading to well-defined changes in gene expression that determines their fate. However, even a given signal can be interpreted in a variety of ways and can elicit a range of outcomes depending on the particular cell type or its position within a cellular field (Wolpert, 1969). Furthermore, a limited number of

signaling pathways are used repeatedly and in different contexts to control organismal development (Gerhart, 1999; Barolo and Posakony, 2002). The ability of the organism to orchestrate an elaborate interplay of cues and cellular factors leading to defined changes in gene expression is essential for its development and viability such that the misregulation of any of the underlying processes is detrimental and can result in disease.

1.2 REGULATION OF BACTERIAL GENE EXPRESSION

A single multi-subunit RNA Polymerase enzyme is responsible for the transcription of genes in bacteria (Ebright, 2000). The core RNA Polymerase enzyme consists of multiple subunits ($\beta\beta'\alpha_2\omega$) and is similar in structure to the eukaryotic RNA Polymerase II, however the former must recruit a σ subunit to form the RNA Polymerase holoenzyme that is then targeted to specific promoters (Fu et al., 1999; Zhang et al., 1999; Browning and Busby, 2004). The σ subunit is required for bacterial RNA Polymerase activity as it enables its recruitment to specific promoter sequences and facilitates the unwinding of the DNA duplex at the transcription start site. Most bacteria possess many σ proteins that allow recognition of distinct sets of promoters by the RNA Polymerase core enzyme (Browning and Busby, 2004). Once recruited to target promoters the RNA Polymerase holoenzyme forms a closed complex with the DNA, followed by formation of an open complex by melting or the unwinding of the DNA duplex at the transcription start point. This is followed by promoter escape after formation of the first few phosphodiester bonds, elongation and subsequently termination of RNA synthesis (Krummel and Chamberlin, 1989).

Commonly repression of bacterial gene expression involves the binding of a single *trans*-

acting factor that antagonizes gene expression or a repressor that binds DNA in a sequence specific manner close to or overlapping with the promoter and direct inhibition of the transcription machinery. For example, the LacI repressor binds close to the transcription initiation site, it is brought in close proximity of the promoter by DNA looping utilizing auxiliary sites and blocks the access of the RNA Polymerase to the promoter (Lewis, 1996). Other bacterial repressors, including phage lambda cI repressor and the LexA repressor, also target promoter proximal sites to block RNA polymerase binding (Hawley et al., 1985; Bertrand-Burggraf et al., 1987). Further the DnaA protein of *Escherichia coli* can negatively regulate its own expression from two promoters 1P and 2P by binding specific sites located between them, followed by oligomerization and inhibition of RNA Polymerase access to both promoters (Lee and Hwang, 1997). Also the binding of DnaA protein within the *guaB* gene can also trigger premature termination of transcription (Tesfa-Selase and Drabble, 1996). The bacteriophage ϕ29 represssor protein p4 binds at 5' proximal sites of the viral A2b promoter and interacts with the α subunit of the RNA Polymerase to prevent promoter escape and thereby inhibits transcriptional elongation (Monsalve et al., 1996). The MerR repressor binds the *merT* promoter simultaneously with the RNA Polymerase and blocks the transition from closed to open promoter complex in the absence of mercury (Heltzel et al., 1990). In the presence of mercury MerR undergoes allosteric changes that mediate DNA bending to permit open complex formation (Ansari et al., 1995). Similarly the *Escherichia coli* GalR and KorB repressors also block the closed to open complex transition of the RNA Polymerase bound to the promoter at transcription initiation (Williams et al., 1993). Bacterial repressors like the CytR repressor directly interact with the activator protein, CRP and prevent activation of gene expression (Shin et al., 2001). Finally unlike the more complex eukaryotic repression mechanisms [see section 1.3] bacterial repression rarely, if at all,

employ mechanisms where multiple DNA binding, sequence specific repressors cooperate and recruit additional proteins forming multi-protein complexes that influence transcription.

1.3 REGULATION OF EUKARYOTIC GENE EXPRESSION

Transcription in eukaryotes is a complex process that involves a number of *trans*-acting proteins, including the general transcription factors that act in concert to facilitate the optimum binding of the RNA polymerase II to the immediate upstream promoter elements that may consist of a TATA box or downstream promoter elements (DPE) of genes (Gaston and Jayaraman, 2003). The regulation of eukaryotic gene expression is further complicated because DNA in genomes is packaged into chromatin; the fundamental unit of chromatin, the nucleosome consists of 147 bp of DNA wrapped around a highly conserved histone protein octamer containing two copies each of the core histones H2A, H2B, H3 and H4 (Luger et al., 1997; Li and Reinberg, 2011). This association of DNA with histones hinders its accessibility to RNA polymerase and transcription factors. Further the chromatin structure is highly heterogeneous along the genome and can adopt higher-level three-dimensional arrangements forming complex local structures (Li and Reinberg, 2011). The chromatin structure is also very dynamic, involving the repositioning of nucleosomes as a result of chromatin remodeling (Clapier and Cairns, 2009) or the presence of histone variants (Swaminathan et al., 2005; Doyen et al., 2006). Additionally, covalent modification of the amino terminal ‘tails’ of histones can alter chromatin compaction (Fischle et al., 2003; Shogren-Knaak et al., 2006) and/or the binding of non-histone proteins that can influence transcription (Jacobs and Khorasanizadeh, 2002).

In addition to the general transcription machinery that functions at all genes, the spatial and temporal control of gene expression is achieved by additional *trans*-acting factors called regulatory transcription factors that will bind in a sequence specific manner adjacent to the genes they regulate; regions where groups of regulatory factors bind are known as *cis*-regulatory modules or enhancers and these may be positioned long distances away from target promoters (Geyer et al., 1990; Small et al., 1991; Stanojevic et al., 1991; Ip et al., 1992; Arnone and Davidson, 1997; Lomvardas et al., 2006; Sanyal et al., 2012; Sudou et al., 2012). Regulatory transcription factors may positively or negatively regulate transcription and are known as activators and repressors, respectively. Regulation of gene expression may be refined further by posttranslational modification of regulatory transcription factors affecting their subcellular localization, DNA binding, stability and protein-protein interactions (Lai and Rubin, 1992; Brunet et al., 1999; Chakrabarti et al., 2000; Cowley and Graves, 2000; Tootle et al., 2003; Essers et al., 2004). Primarily regulatory transcription factors function by controlling the chromatin structure - compaction, covalent modification of histones, and nucleosome positioning.

1.3.1 Mechanism of enhancer activity and models of enhancer architecture

As mentioned above enhancers are DNA elements that can regulate transcription by recruiting regulatory transcription factors that will in turn promote or antagonize the assembly of the basal transcription machinery including RNA polymerase II and general transcription factors at target promoters. Enhancers are often positioned at large distances away from their target promoters and may even be situated on different chromosomes (Geyer et al., 1990; Lomvardas et al., 2006). Interactions between enhancers and distant promoters may be mediated by the looping of the

intervening DNA, whereby enhancer bound transcription factors can recruit additional cofactors that promote bending of the intervening DNA to modulate the positioning of nucleosomes. Support for this model comes from experimental evidence obtained with chromosome conformation capture (3C) technique and allied methods that can detect interactions between spatially separated regions of chromosomes in *cis* (intra-chromosomal) or *trans* (inter-chromosomal) (Carter et al., 2002; Dekker et al., 2002). Long range interactions between enhancers and promoters have been supported by chromatin based studies in the vertebrate *β-Globin, H19, Igf2, Myb* loci and the *Abdominal B* (*Abd B*) regulatory element in *Drosophila* (Tolhuis et al., 2002; Murrell et al., 2004; Sipos and Gyurkovics, 2005; Degner et al., 2011; Stadhouders et al., 2012).

Transcription factors can also inhibit loop formation as demonstrated for the *HoxA* locus where OCT4 can block loop formation by antagonizing cohesin binding (Kim et al., 2011); the cohesin complex mediates loop formation at various loci (Nativio et al., 2009; Kagey et al., 2010; Degner et al., 2011). In *Drosophila* the repressor Snail prevents looping and antagonizes enhancer-promoter interaction (Chopra et al., 2012).

Enhancers contain binding sites for several transcription factors usually both activators and repressors, often with multiple binding sites for each. The specific recruitment of individual factors to enhancers allows the expression of target genes to be refined on the basis of the cellular context, developmental stage, timing and extracellular cues (He et al., 2012). For example, the same regulatory elements in the *Drosophila nidogen* gene have been shown to bind different forkhead domain-containing transcription factors in different tissues with distinct regulatory outputs (Zhu et al., 2012). In contrast, multiple homeobox (Hox) transcription factors

with very similar DNA binding potencies can target the same gene via different enhancers in different tissues (Enriquez et al., 2010).

Further the transcriptional output from an enhancer involves the cooperative integration of inputs, so that the activation of the expression of a gene in a particular tissue or at a precise developmental stage is often the outcome of the cooperative binding of multiple activators including ones that are cell-type, stage and signal specific to maintain a tight regulatory control. For example, in *Drosophila* output from a Hox-targeted enhancer of the *reaper* gene requires not only the Hox protein Deformed (Dfd) but also eight additional transcription factors (Stobe et al., 2009).

Three different models have been proposed to describe enhancer architecture and how the combinatorial logic code provided by different regulatory factors maybe integrated by them, these are: the enhanceosome, the bill-board and the transcription factor collectives models (Arnosti and Kulkarni, 2005; Junion et al., 2012). While the enhanceosome model postulates that the cooperative binding of a group of transcription factors using a precise arrangement of binding sites in the DNA is necessary for enhancer activity, the billboard model hypothesizes that enhancers are very flexible and while each *cis*-regulatory sub-element needs to be bound to a corresponding regulatory protein the relative orientation and spacing between them and other sub-elements does not contribute to overall enhancer function (Arnosti and Kulkarni, 2005). The transcription factor collectives model predicts that transcription factors are recruited cooperatively to enhancers but without a strict grammar of regulatory motifs and that binding sites need not be present for each transcription factor present at the enhancer such that these maybe bound indirectly by protein-protein interactions (Junion et al., 2012).

1.3.2 Transcriptional Activation

Transcription initiation involves a set of general/basal transcription factors including TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH that will assemble on the core promoter to form the preinitiation complex (PIC) directing the RNA polymerase II (Pol II) to the transcription start site. Gene activation involves the stepwise recruitment of several multi-subunit regulatory complexes including the highly conserved Mediator complex. *Drosophila* Mediator has no perceptible effect on basal transcription, at least in an *in vitro* system (Park et al., 2001; Gu et al., 2002) but interacts with Pol II and the general transcription factors facilitating the assembly of the PIC at the transcription start site (Yudkovsky et al., 2000). In addition, activation of transcription involves regulatory transcription factors or activator proteins that bind precise *cis*-regulatory sequences at target genes with their DNA binding domain and also possess a separable activation domain to stimulate transcription (Ptashne and Gann, 1997; Gaston and Jayaraman, 2003). Activator proteins may function by promoting PIC assembly via interaction with one or more components of the basal transcription machinery and sub-units of the Mediator complex (Ge et al., 2002; Bhaumik et al., 2004), or may facilitate subsequent steps such as transcription elongation and reinitiation (Fig. 1).

Primarily activators function by recruiting auxiliary proteins known as co-activators that lack DNA binding activity (Fig. 1). Activators and co-activators often promote transcription by the recruitment of chromatin modifying proteins, including histone-modifying enzymes such as histone acetyltransferases that covalently modify histone tails leading to chromatin decondensation (Ogryzko et al., 1996; Akimaru et al., 1997). Histones H3 and H4 are typically acetylated at active genes and the highest level of acetylation coincides with the promoter and 5' regions of the gene (Kristjuhan et al., 2002; Liang et al., 2004; Liu et al., 2005). Furthermore

chromatin remodelers such as switch/sucrose nonfermentable (SWI/SNF) and chromodomain helicase DNA binding protein 1 (CHD1) are associated with active loci in *Drosophila*; these proteins facilitate repositioning and ejection of nucleosomes (Stokes et al., 1996; Armstrong et al., 2002). Thus, the chromatin modifying activities of the activator and allied co-activator machinery results in the expansion of the nucleosome free regions making genes more accessible to additional activators or to the basal transcriptional machinery.

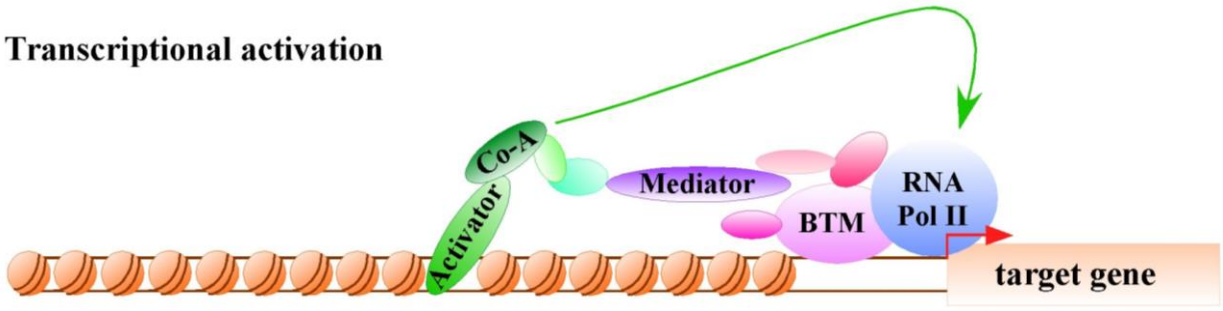

Figure 1: Overview of transcriptional activation

Activators function largely by recruiting accessory proteins, co-activators (Co-A) that in turn may function via modification of chromatin structure to facilitate recruitment of the RNA polymerase II and components of the basal transcription machinery (BTM) and promote initiation of transcription.

1.3.3 Transcriptional Repression

Precise spatiotemporal patterns of gene expression that are vital for development and pattern formation may be achieved by precisely regulating the localization and/or activity of activators, but largely this is not the case and activators that are required for expression of differentiation-specific genes are usually expressed in much wider domains than their targets. The spatial domains/boundaries of gene expression are often established by modification of the localization or activity of transcriptional repressors, more specifically by the absence of repressors. Essentially, genes are expressed because activators are present but also because a repressor is not. Thus, repressors are indispensible for appropriate regulation of gene expression (Gray and Levine, 1996) and their absence results in ectopic expression of many genes.

1.3.3.1 Mechanisms of repression: simple competition

Repressors may function by competing with activators for the same or overlapping binding sites thus preventing their recruitment to target promoters (Fig. 2). However, the general applicability of this binding site competition model is uncertain because most repressor binding sequences do not overlap with activator sites (Arnosti et al., 1996) and *in vivo* data to support this model are largely lacking.

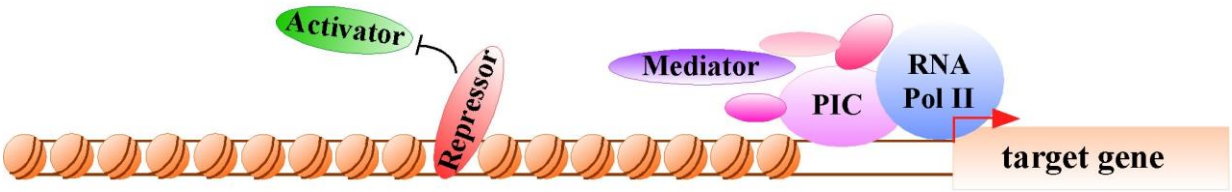

Figure 2: Repression by simple competition

Repressor activity maybe dependent on their DNA binding domain and they function by competing with activators for the same or overlapping binding sites, blocking the productive assembly of the pre-initiation complex (PIC) thereby antagonizing transcription initiation at the target gene promoter.

1.3.3.2 Mechanisms of repressions dependent on repression domain(s)

In addition to their DNA binding domain almost all repressors that have been studied in any detail have been shown to possess regions outside of the DNA binding domain that have been termed 'repression' domains; a repression domain being defined as a region that can confer repressive activity to a heterologous DNA binding domain (Hanna-Rose and Hansen, 1996). The mechanism of activity of repression domains may be classified as: (a) non-chromatin based and (b) chromatin based.

(a) Non-chromatin based repression mechanisms include the following:

(i) Inhibition of activator or basal transcriptional machinery activity. This could involve direct protein-protein interactions between the repressor and activator or a general transcription factor (Fig. 3A). For example, in both yeast and mammals the Down-regulator of transcription 1 (Dr1) repressor, also known as Negative Cofactor 2β (NC2β) directly binds the TATA-binding protein (TBP) and antagonizes RNA Polymerase II activity *in vitro* and *in vivo* (Inostroza et al., 1992; Gadbois et al., 1997; Kim et al., 1997; Yeung et al., 1997). There is also good evidence that

some viral proteins, such as the E1A protein of Adenovirus, can directly inhibit the basal transcription machinery by binding a specific component (Song et al., 1995). The switch/sucrose nonfermentable (SWI/SNF) enzyme, modifier of transcription 1 (Mot1) in yeast and BTAF1 in humans can displace TBP from target promoters and downregulate gene expression (Wollmann et al., 2011; Moyle-Heyrman et al., 2012). However, these factors are generally not standard DNA-binding transcription factors and access the promoters of genes by binding other transcription factors. For example, the repressive activity of Dr1 is dependent on its TBP binding domain that tethers it to the promoter along with a glutamine and alanine rich domain (QA) and is stimulated by dimerization with the cofactor Dr1 associated protein 1 (DRAP1) or Negative Cofactor 2α (NC2α) (Goppelt et al., 1996; Kim et al., 1997; Yeung et al., 1997; Kamada et al., 2001). Also Mot1 activity is dependent on its N-terminal TBP binding region and C-terminal SWI/SNF ATPase domain (Wollmann et al., 2011). It is has been suggested that 'repression domains' from standard regulatory transcriptional repressors can function by inhibiting the basal transcriptional machinery. However, there is very little direct evidence for this. One of the few exceptions is the Even-skipped protein of *Drosophila*, which was shown to possess a repression domain that could bind TBP and inhibit transcription in transient transfections (Um et al., 1995). However, this study needs to be readdressed *in vivo* as more recent studies indicate this repression domain recruits the co-repressor, Atrophin (Zhang, S. et al., 2002).

(ii) 'Loop-dependent' repression. If a repressor can interfere with the basal transcriptional machinery it must obviously be brought to the promoter to do so. In theory, if such repressors are bound to distal enhancers they would have little opportunity to influence transcription. However, as mentioned above [see sub-section 1.3.1], such enhancers are also bound by activators, which communicate with the promoter via DNA looping. This looping may bring

repressors in close proximity of the promoter, allowing the repressor to interfere with the basal transcription machinery (Fig. 3B) (Chopra et al., 2012). There is little evidence, as yet, to support this idea.

(iii) Anti-looping. As mentioned above, repressors may also employ an anti-looping mechanism to repress target genes, whereby, they antagonize factors required for looping and block any communication between an enhancer and the promoter (Fig. 3C) (Chopra et al., 2012). This repression mechanism may be utilized at 'poised' genes that need to be rapidly up-regulated in course of development and have stalled RNA polymerase II at their promoter, as the repressor can efficiently block transcription without influencing the basal transcription machinery (Chopra et al., 2012).

(b) Chromatin based mechanisms of transcriptional repression involve the modulation of chromatin structure, although there is little evidence that repression domains may do this directly there is strong evidence that they do this indirectly, by recruiting co-repressors (Fig. 3A) as is discussed below [see sub-subsection 1.3.3.3]. In addition, the repressed state of genes may be maintained through many cell divisions and a key effector of this epigenetic silencing is the Polycomb group (PcG) proteins [see sub-subsection 1.3.3.4].

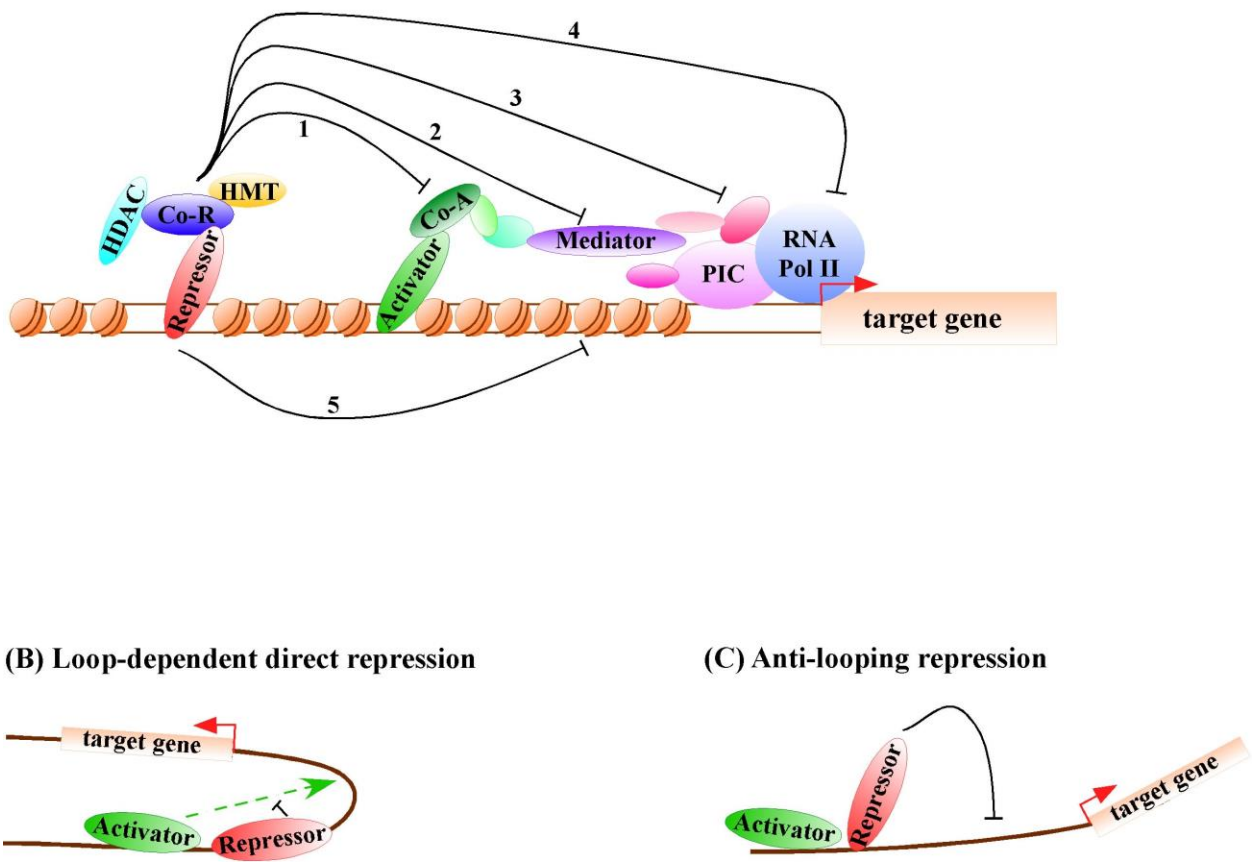

Figure 3: Mechanisms of transcriptional repression.

(A) The activity of repressors may be dependent on their repression domain(s). These often contain short peptide motifs allowing the recruitment of auxiliary proteins, co-repressors (Co-R) that can antagonize transcription in several ways: (1-4) Antagonizing the recruitment and interfering with the activity of co-activators (Co-A), one or more components of the preinitiation complex (PIC), the Mediator complex or RNA polymerase II (non-chromatin based mechanism of repression). (5) Modification of chromatin structure and compaction through their intrinsic activity or by recruitment of additional chromatin modifiers such as histone deacetylases (HDACs) and histone methyltransferases (HMTs) (chromatin based mechanism of repression). (B) Repressors may function in a loop dependent mechanism whereby they bind to distal enhancers, that when also bound to activators come in close vicinity of the basal transcription machinery due to the looping of the intervening DNA, allowing them to antagonize transcription. (C) Repressors may also function by the anti-looping mechanism that entails blocking the looping of DNA to prevent activators from activating transcription.

1.3.3.3 Co-repressors

The term co-repressor may refer to a single protein or a scaffolding protein that mediates the assembly of a complex of proteins facilitating repression at target genes when recruited by repressor proteins (Payankaulam et al., 2010). Repressors possess short peptide sequences often contained within their repression domains that mediate recruitment of specific co-repressors. Co-repressors may either possess intrinsic activity or may recruit additional proteins including histone modifying enzymes such as histone deacetylases, histone methyltransferases, lysine demethylases that may add or remove specific covalent modifications that may impact chromatin compaction or the binding of non-histone proteins critical for transcription (Fig. 3A). In addition, co-repressors may recruit ATP utilizing chromatin remodeler complexes that aid in organizing regions of DNA in condensed nucleosomal arrays restricting its access to activators, the basal transcription machinery and attract additional chromatin modifiers to reinforce repression. For example, the vertebrate Mi2β/NuRD co-repressor complex combines chromatin remodeler ATPases, histone deacetylases and lysine specific histone demethylase 1 (Wang et al., 2009). Co-repressors may also act by 'quenching' that is directly interfering or displacing co-activators such as CBP/p300 histone acetyltranferases (Kim, J. H. et al., 2005).

1.3.3.4 Epigenetic silencing by Polycomb proteins

Polycomb group (PcG) proteins globally enforce long-term, heritable or epigenetic silencing of repressed genes and were first identified in *Drosophila* as repressors of the homeotic (Hox) genes (Lewis, 1978; Struhl, 1981; Breen and Duncan, 1986). Further genome-wide studies revealed hundreds of other PcG targets in *Drosophila* (Negre et al., 2006; Schwartz et al., 2006; Tolhuis et al., 2006) and mammalian cells (Boyer et al., 2006; Bracken et al., 2006; Lee et al., 2006). The PcG machinery is a critical regulator of epigenetic silencing phenomena including

parent-of-origin imprinting and X-chromosome inactivation; additionally it has been implicated in transcriptional regulation of embryonic, adult stem cells and the gene expression circuitry gone awry in cancer cells (Boyer et al., 2006; Ezhkova et al., 2009; Simon and Kingston, 2009; Ernst et al., 2010; Ntziachristos et al., 2012).

In *Drosophila* the Polycomb response elements (PREs) at target genes bind *trans*-acting factors that in turn will directly or indirectly mediate the recruitment of PcG proteins. In mammals sequences enriched in CpG dinucleotides (CpG islands) and long noncoding RNAs are known to target PcG proteins, in addition to *Drosophila* PRE-like DNA elements (Rinn et al., 2007; Ku et al., 2008).

PcG complexes can function by covalent modification of histone 'tails' resulting in chromatin signatures like the histone H3 Lys 27 trimethylation (H3K27me3) that concur with the distribution of PcG complexes and are though to mediate PcG silencing (Cao et al., 2002; Czermin et al., 2002; Muller et al., 2002).

1.3.4 Promoter proximal pausing of RNA Polymerase II

Genome-wide studies have revealed that RNA Polymerase II is actually present – but 'paused' - at the promoter of genes that are not actually being transcribed. At these loci the preinitiation complex (PIC) has been assembled but the release of RNA polymerase II is precisely regulated. This appears to be important in the modulation of transcriptional output in response to developmental and environmental cues in metazoans and has been demonstrated at large numbers of genes including those involved in development, cell proliferation, differentiation, stress or damage responses in *Drosophila* and mammalian cells (Kim, T. H. et al., 2005; Guenther et al., 2007; Muse et al., 2007; Zeitlinger et al., 2007; Lee et al., 2008). Pausing of

RNA polymerase II in early transcriptional elongation occurs as a result of the association of the pause-inducing factors, DRB-sensitivity-inducing factor (DSIF) and negative elongation factor (NELF) with the elongation complex (Wada et al., 1998; Yamaguchi et al., 1999). Pausing of RNA polymerase II is relieved by the recruitment of the positive elongation factor (P-TEFB) that phosphorylates the DSIF-NELF repressive complex causing the dissociation of NELF and alteration of DSIF confirmation such that it promotes transcriptional elongation leading to productive transcriptional elongation (Marshall and Price, 1992; Marshall and Price, 1995; Peterlin and Price, 2006).

Promoter proximal pausing allows genes to be potentiated for rapid activation in response to environmental cues; for example, paused RNA polymerase II has been detected on heat shock genes (Boehm et al., 2003). Further paused RNA polymerase II has been demonstrated at many developmental control genes in the *Drosophila* embryo that are induced synchronously. The depletion of NELF which antagonizes early transcriptional pausing results in embryonic lethality in mice and *Drosophila* underscoring the importance of promoter proximal pausing in developmental processes (Wang et al., 2007; Zeitlinger et al., 2007; Amleh et al., 2009; Boettiger and Levine, 2009; Wang et al., 2010). Genome-wide studies have also shown that genes with paused RNA polymerase II have low levels of nucleosome occupancy at their promoters making these more accessible to the general transcription machinery, while the knockdown NELF resulted in an increase in promoter nucleosome occupancy of these genes (Gilchrist et al., 2008; Gilchrist et al., 2010). Finally, many genes with paused RNA polymerase II encode for constitutively expressed components of signal transduction pathways, thus promoter proximal pausing is limited not only to prime genes for rapid induction but plays a broader role in modulation of cellular responses to external stimuli (Gilchrist et al., 2012).

1.4 SHORT- AND LONG-RANGE REPRESSION

Repressors may be classified as short- or long-range factors based on the range of their activity and whether they can antagonize proximally or distally located activators (Gray and Levine, 1996). Short-range repressors act locally at distances less than 100-150 bp to antagonize adjacent activators while not affecting more distantly bound ones (Fig. 4A) (Gray and Levine, 1996). The pair-rule genes *even-skipped* and *hairy* are expressed in seven transverse stripes along the anterior-posterior (AP) axis of the early *Drosophila* embryo. The spatial regulation of their expression is achieved by short-range repressors encoded by gap genes such as *giant, hunchback, krüppel* and *knirps* (Pankratz et al., 1990; Small et al., 1991; Arnosti et al., 1996; Small et al., 1996). These factors work through the presence of several autonomous enhancers in their target genes (Akam, 1989). An individual enhancer often directs the expression of one or two stripes, but since the enhancers function independently of one another to direct expression at different locations along the AP axis, so that the characteristic seven-stripe expression pattern can be produced by an appropriate combination of enhancers at a locus. These enhancers can be situated fairly close to each other, and so it is essential that repressors bound at one region to inhibit expression of one stripe do not interfere with an adjacent enhancer to which they do not bind. The ability of these enhancers to act independently is highly dependent on the ability of the repressors recruited by them to function locally and not elicit effects over longer distances. The distance requirements of short-range repressors such as Knirps, Snail, Giant and Kruppel was investigated by generating fusion constructs in which the distance between repressor and activator binding sites was increased to more than 100 bp; this alleviated their repressive activity confirming that they exert short-range/local activity to antagonize gene expression (Small et al., 1991; Hoch et al., 1992; Small et al., 1993; Arnosti et al., 1996). Studies with several short-range

repressors have also provided insights into chromatin structure alterations that accompany short-range repression, including a local increase in histone density, deacetylation, loss of activator occupancy at target enhancers and no effect on recruitment of the basal transcription machinery (Zeitlinger et al., 2007; Qi et al., 2008; Li and Arnosti, 2011). A number of short-range repressors in the early *Drosophila* embryo appear to partly utilize a common co-repressor, C-terminal Binding Protein (CtBP) (Nibu et al., 1998a).

Long-range repressors such as the *Drosophila* protein Hairy induce large-scale repression by inhibiting activators at enhancers located several thousand base pairs away from where the repressor is bound (Barolo and Levine, 1997; Jimenez et al., 1997). Hairy recruits Groucho (Gro) and the latter has thus been implicated in global silencing of target genes to which it is recruited by acting at over 1kb (Fig. 4B) (Jimenez et al., 1997). Long-range repressors when recruited appear to repress by causing large-scale histone deacetylation, directly or indirectly blocking RNA polymerase II recruitment but do not affect activator occupancy (Dearolf et al., 1989; Janssens et al., 2006; Li et al., 2008; Martinez and Arnosti, 2008; Li and Arnosti, 2011).

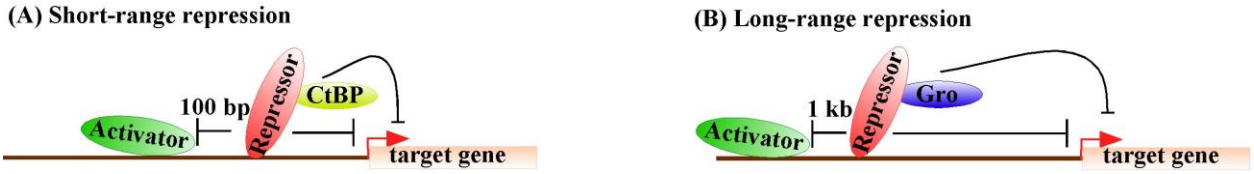

Figure 4: Short- and Long-range models of transcriptional repression

(A) Co-repressors such as CtBP that silence enhancers over a distance of 100 bp or less and are thought to facilitate short-range repression. (B) Groucho (Gro) when recruited by repressors can silence transcription over larger distances of 1 kb and executes long-range repression.

1.5 CO-REPRESSORS IN DROSOPHILA

1.5.1 CtBP

The C-terminal binding protein (CtBP) family of co-repressors is conserved in metazoa and regulates a variety of processes essential for development and survival. The first member of this family, CtBP1 was identified as a protein that interacted with the C-terminus of human adenovirus E1A protein (Boyd et al., 1993; Schaeper et al., 1995). Unlike in vertebrates a single gene encodes for CtBP in the *Drosophila* genome. CtBP proteins are homologous to NAD-dependent D hydroxyacid dehydrogenases and are structurally very similar to these enzymes (Kumar et al., 2002; Nardini et al., 2003). Like dehydrogenases they function as homodimers, where each CtBP subunit contains a nucleotide-binding domain that binds NAD/NADH (Fig. 5A). In addition, each CtBP subunit contains a substrate binding domain that interacts with a short, conserved PxDLS sequence motif (where x can be any amino-acid), originally identified in the adenovirus EA1 protein and present in transcriptional repressors interacting with CtBP (Schaeper et al., 1995; Turner and Crossley, 1998; Nardini et al., 2003) (Fig. 5A). In addition to the PxDLS motif certain transcription factors also possess a second redundant binding motif, RRT to mediate their interaction with CtBP (Quinlan et al., 2006) (Fig. 5A). Since several RRT motif containing transcription factors also possess one or more PxDLS motifs, the former is though to serve to stabilize their interaction with CtBP cofactors (Zhang, C. L. et al., 2001). Interestingly the neuronal repressor NRSF/REST that lacks prototypical CtBP interacting motifs can still directly interact with it (Garriga-Canut et al., 2006). In the *Drosophila* embryo, several short-range repressors that act at a distance of 100-150 bp to antagonize activators are dependent on the recruitment of the CtBP for their activity (Fig. 4A) (Nibu et al., 1998a).

CtBP proteins appear to function by the recruitment of chromatin modifying factors such as histone deacetylases, histone methyltransferases and histone demethylases (Shi et al., 2003; Struffi and Arnosti, 2005). In addition, the repressive activity of CtBP has also been shown to be dependent on its NAD binding (Sutrias-Grau and Arnosti, 2004). CtBP proteins can also facilitate the recruitment of the Polycomb group proteins [see sub-subsection 1.3.3.4] in both vertebrates and in *Drosophila* that mediate long term heritable gene silencing (Atchison et al., 2003; Lund and van Lohuizen, 2004; Srinivasan and Atchison, 2004); however this has been shown to be dependent on the levels of CtBP (Basu and Atchison, 2010).

While CtBP proteins are ubiquitously expressed their activity and subcellular localization may be modulated in response to cues from their microenvironment eliciting large-scale changes in gene expression profiles. For example the SUMOylation of mammalian CtBP1, acetylation of CtBP2 by p300 and phosphorylation of CtBP1 by p21activated kinase modulates their nuclear retention and repressive activity (Lin et al., 2003; Zhao et al., 2006). In addition, the transducer β-like proteins, TBL1 and TBLR1 mediate ubiquitylation dependent degradation of mammalian CtBP1 and CtBP2 when recruited to NCoR and SMRT repressor complexes (Perissi et al., 2008). Like its vertebrate counterpart the *Drosophila* CtBP gene can encode for multiple protein isoforms (Sutrias-Grau and Arnosti, 2004) but no evidence has been found as yet for any differences in their activity or their modulation by posttranslational modification.

1.5.2 Groucho

Groucho (Gro) proteins of *Drosophila* and its vertebrate homolog Transducin-Like Enhancer of split (TLE) and Groucho related gene (Grg) control several key developmental and post-embryonic processes in a variety of organisms by facilitating the repressive activity of a many

transcriptional repressors (Paroush et al., 1994; Dehni et al., 1995). There is a single Gro protein in *Drosophila* in contrast to multiple forms in most vertebrate species (Bajoghli, 2007).

Gro proteins possess five regions defined by their evolutionary conservation: Q, GP, CcN, SP and WD (Fig. 5B). The Q and WD domains are the most highly conserved features of the Gro/TLE family of co-repressors. Sequences within the glutamine rich Q domain facilitate oligomerization of Gro proteins and binding to transcription factors like LEF1, TCF, FoxA and c-Myc (Chen et al., 1998; Song et al., 2004; Daniels and Weis, 2005; Orian et al., 2007). The glycine-proline rich GP region may be involved in interaction of Gro with histone deacetylases and the CcN region contains a nuclear localization signal (Stifani et al., 1992; Chen et al., 1999). While GP, CcN and the serine-proline rich SP region are poorly conserved they have been shown to be critical for viability, mediating repression and ensuring target gene specificity (Turki-Judeh and Courey, 2012). The WD domain mediates interaction of Gro with short peptide motifs in transcription factors that fall into two categories: a WRPW and related tetrapeptide motifs or an octapeptide engrailed homology 1 (eh1) motif FxIxxIL (where x can be any amino acid) (Paroush et al., 1994; Fisher et al., 1996; Jimenez et al., 1997; Goldstein et al., 2005). When bound to Gro proteins the WRPW motif forms a compact structure that is distinct from the helical conformation adopted by eh1 motif on the co-repressor surface (Jennings et al., 2006). Putative phosphorylation sites are located in the SP region and WD domain (Fig. 5B) (Hasson et al., 2005).

Long-range repressor proteins such as Hairy are dependent on Gro for silencing enhancers located several kilobases away from the repressor binding site (Fig. 4B) (Paroush et al., 1994; Jimenez et al., 1997). This long-range repression via Gro has been attributed to its ability to oligomerize and make extensive contacts by ‘spreading’ along the target locus or

alternatively, according to the 'turban' model, it may remain tethered to the promoter regions and form large multi-protein complexes, around which the proximal and distal ends of the target gene are wrapped (Jennings et al., 2008; Martinez and Arnosti, 2008). However, Gro has also been shown to mediate only short-range repression in some contexts (Payankaulam and Arnosti, 2009). Why Gro functions over a short range in some situations but long range in others is not understood.

Gro functions by modulation of chromatin structure and compaction and to this end it has been shown to recruit chromatin-modifying proteins like the histone deacetylase, Rpd3 in *Drosophila* (Chen et al., 1999; Winkler et al., 2010).

Gro proteins are ubiquitously expressed throughout *Drosophila* development, and similarly the vertebrate TLE proteins are also largely expressed in most tissues (Delidakis et al., 1991; Stifani et al., 1992; Dehni et al., 1995; Leon and Lobe, 1997; Molenaar et al., 2000). But Gro/TLE proteins are also subjected to a variety of posttranslational modifications that provide additional layers for fine-tuning gene expression. Gro activity can be downregulated in several ways including phosphorylation by Receptor Tyrosine kinase pathways and Homeodomain-interacting protein kinase 2 (Choi et al., 2005; Hasson et al., 2005; Cinnamon et al., 2008). In contrast, the phosphorylation of vertebrate TLE1 by casein kinase II increases its repressive activity and binding with the Hairy homolog, Hes1 (Nuthall et al., 2002; Buscarlet et al., 2009). Also the small ubiquitin-like modifier 1 (SUMO-1) protein is conjugated with Gro at multiple lysine residues in the GP and SP regions (Fig. 5B); sumoylation enhances Gro interaction with histone deacteylase 1 (HDAC1) which is also SUMO modified and thereby facilitates Gro repressive activity (Ahn et al., 2009). Further the poly(ADP-ribose) polymerase 1 (PARP1) mediated polyADP ribosylation promotes the ejection of TLE1 proteins from promoters and the

derepression of target genes (Ju et al., 2004). In addition, dominant-negative forms of TLE/GRG proteins have been identified in vertebrates that include the Amino-terminal Enhancer of Split (AES) or the GRG5 proteins that antagonize the repressive activity of the TLE/GRG co-repressor proteins by multimerizing with them and disabling transcription factor binding or formation of productive repressive complexes at target promoters (Roose et al., 1998; Marcal et al., 2005; Allen et al., 2006).

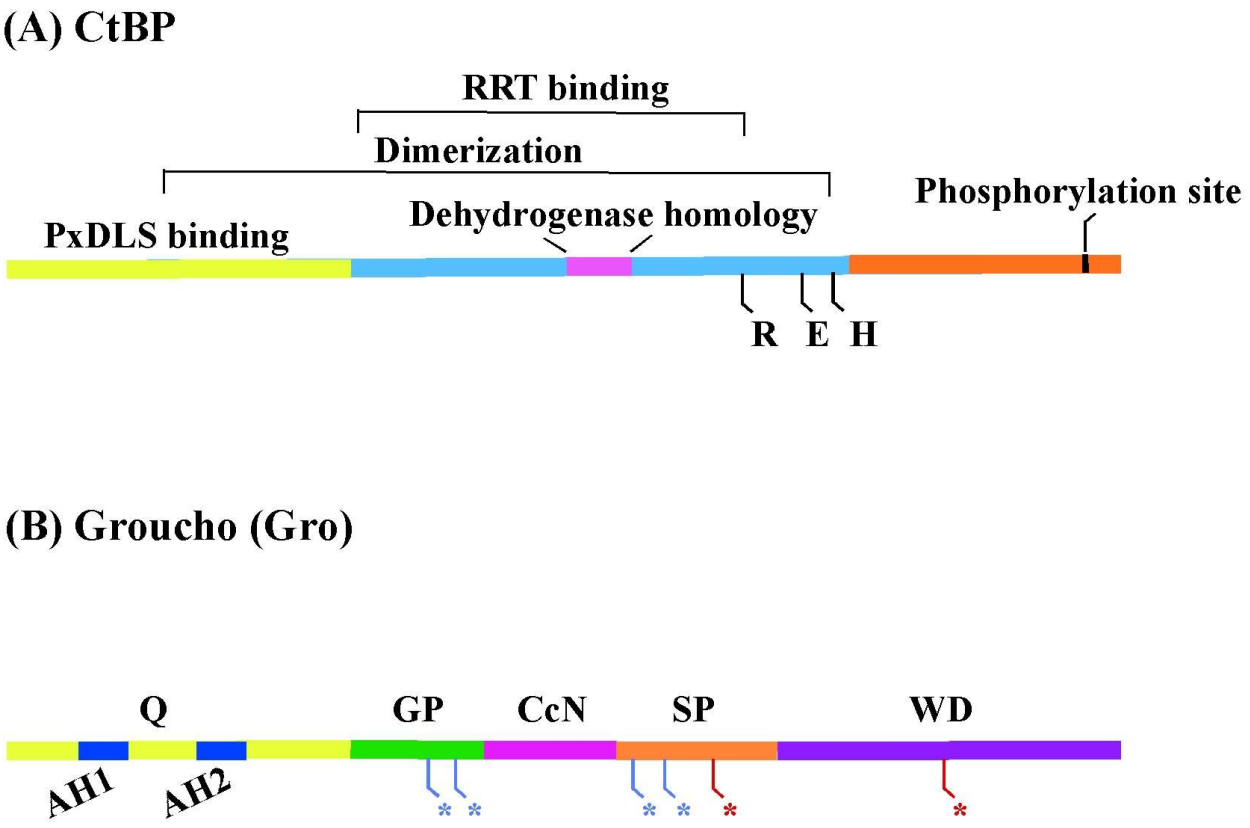

Figure 5**: Domains within CtBP and Groucho (Gro) proteins.**

(A) CtBP proteins contain an N-terminal substrate-binding domain that includes a PxDLS binding cleft. The dimerization interface and the region that forms the RRT binding cleft has also been indicated. A central domain shows significant homology to NAD dependent dehydrogenases; residues that mediate NAD(H) binding and catalytic activity (R, E, H) have been shown. Residues that are phosphorylated on the unstructured C-terminal region have also been shown. (B) Groucho (Gro) proteins contain an N-terminal glutamine-rich region, Q containing two amphipathic α helices, AH1 and AH2 and mediate oligomerization and binding with transcription factors. The central poorly conserved region includes a glycine-proline rich region, GP that mediates interaction with histone deacetylases, the CcN region that contains the nuclear localization signal, the serine-proline rich region, SP containing sumoylation and phosphorylation sites (indicated with blue and red asterix respectively). Phosphorylation sites are contained in the SP region and WD domain and have been indicated with red asterix (*). Sumoylation sites are present in the Gro GP and SP region and have been indicated with blue asterix (*). The C-terminal WD domain enables binding to transcription factors. Both the Q and WD domain are highly evolutionarily conserved.

1.5.3 Additional examples of co-repressors in *Drosophila*

1.5.3.1 Atrophin

Atrophin (Atro) protein of *Drosophila* belongs to a conserved family of nuclear receptor co-repressors that also contains the vertebrate homologs, Atrophin 1 (ATN1) and RERE/Atrophin 2 proteins (ATN2) (Erkner et al., 2002; Zhang, S. et al., 2002). Atro appears to be required for the repressive activity of Even-skipped, Huckebein and Tailless transcription factors (Erkner et al., 2002; Zhang, S. et al., 2002; Wang et al., 2006; Wehn and Campbell, 2006). Structurally, Atro consists of the N-terminal ELM2 and SANT conserved domains. These domains have been shown to recruit histone deacetylases (HDAC1/2) and histone methyltransferases, G9a respectively, suggesting a role of these domains in repression (Wang et al., 2006; Wang et al., 2008). The C-terminal region of Atro mediates interaction with transcription factors possibly through a highly conserved 16 residue ‘Atro box’ (Zhang, S. et al., 2002; Wang et al., 2006). Atro is a multifunctional protein; in addition to its role as a co-repressor it also functions as a

positive regulator of Hox gene expression and in the cytoplasm is involved in the establishment of planar cell polarity (Zhang, S. et al., 2002; Fanto et al., 2003; Kankel et al., 2004).

1.5.3.2 SMRTER

The Silencing Mediator of Retinoic and thyroid hormone receptors (SMRT) family of co-repressors includes the vertebrate SMRT and Nuclear Hormone Receptor Co-repressor (N-CoR) co-repressors. The *Drosophila* SMRT-related and Ecdysone receptor interacting factor (SMRTER) protein is analogous to N-CoR and SMRT and shows some conservation of function with them (Chen and Evans, 1995; Horlein et al., 1995; Tsai et al., 1999). Like its vertebrate counterparts the functions of the SMRTER co-repressor complex are not restricted to nuclear receptor regulatory pathways alone and it has been additionally shown to antagonize Notch pathway in patterning the ovary and the wing (Heck et al., 2012). SMRTER has been found to bind the *Drosophila* homologs of the Sin3A co-repressor, transducing-β like 1X-linked proteins (TBL1) or Ebi and histone deacetylases (Tsai et al., 1999; Pile and Wassarman, 2000; Pile et al., 2002; Tsuda et al., 2002).

1.5.3.3 Sin3

The evolutionarily conserved Sin3 co-repressor complex mediates a variety of developmental and growth processes in eukaryotes. The *Drosophila* Sin3 complex primarily recruits histone deacetylases to mediate repression when recruited by transcription factors such as p53, ELK1 and nuclear hormone receptors (Payankaulam et al., 2010). Alternatively the Sin3 complex may be indirectly targeted through its interaction with other co-repressor complexes, for example the SMRTER complex that in turn is recruited by transcription factors (Horlein et al., 1995). In addition to histone deacetylases, SWI/SNF chromatin remodelers, histone methylases, DNA

methylases and protein glycosylases are often found associated with Sin3 complexes (Payankaulam et al., 2010). While a single *Drosophila* gene encodes for Sin3, several alternatively spliced isoforms of it have been isolated that are expressed variably in a developmental stage and tissue-specific fashion (Sharma et al., 2008).

1.5.3.4 Mi-2/NuRD complex

The Mi-2 protein is a conserved ATP dependent chromatin remodeler of the chromodomain-helicase DNA binding (CHD) family. *Drosophila* Mi-2 like its vertebrate homolog associates with the nucleosome remodeling and deacetylation (NuRD) co-repressor complex that also includes histone deacetylases, negatively regulating transcription and controlling important cell fate specification roles in all of metazoa. For example it participates in the transcriptional repression of Hox genes by Hunchback and Polycomb proteins in *Drosophila* (Kehle et al., 1998). Additionally Mi-2 is also found associated with another chromatin remodeler complex, the *Drosophila* specific MEP-1 containing complex (Mec) involved in transcriptional repression (Murawsky et al., 2001; Kunert et al., 2009). Further the SUMOylation of transcription factors has been shown to enhance the recruitment of Mi-2 containing complexes (Stielow et al., 2008; Reddy et al., 2010). In addition to its roles in mediating repression the *Drosophila* Mi-2 also co-localizes with the elongating form of RNA Polymerase II at actively transcribed genes on polytene chromosomes and is recruited to sites of transcription upon heat shock (Murawska et al., 2011; Mathieu et al., 2012).

1.6 CO-REPRESSORS IN VERTEBRATES

As mentioned above most co-repressors in *Drosophila* show variable degrees of evolutionary conservation in structure and/or function resulting in homologous or analogous counterparts in vertebrates [see section 1.5]. Table 1 lists some co-repressors known to function in vertebrates. As discussed above co-repressors in vertebrates also primarily function as adaptors recruiting chromatin-modifying enzymes that modulate chromatin structure, compaction thus interfering with the activity and accessibility of proteins involved in transcription. Further vertebrate co-repressors are also subject to a variety of posttranslational modifications that regulate their subcellular localization and activity, as discussed above for CtBP and Gro proteins in *Drosophila* [see subsection 1.5.1 and 1.5.2] and serve as additional layers of regulation fine tuning the transcriptional output of a number of signaling pathways in response to changes in their microenvironment.

1.6.1 SMRT and NCoR

The Silencing Mediator of Retinoic and thyroid hormone receptors (SMRT) and Nuclear Hormone Receptor Co-repressor (N-CoR) are paralogous vertebrate proteins that mediate the repressive activity of unliganded nuclear receptors and several transcription factors (Chen and Evans, 1995; Horlein et al., 1995; Lutterbach et al., 1998; Ahmad et al., 2003; Jayne et al., 2006). They recruit histone deacetylases HDAC1, HDAC3, HDAC4, HDAC7 and Sirt1 for mediating their repressive activity (Heinzel et al., 1997; Nagy et al., 1997; Kao et al., 2000; Picard et al., 2004). Both SMRT and N-CoR have two conserved motifs, ID1 and ID2 characterized by an amphipathic core φxxφφ (where φ is a hydrophobic amino acid and x is any

amino acid) flanked by additional sequences, which mediate interaction with nuclear receptors (Hu and Lazar, 1999; Perissi et al., 1999). Further MAPK activity has been shown to phosphorylate and lead to the nuclear export and inactivation of the SMRT co-repressor complex (Hong and Privalsky, 2000).

1.6.2 CoREST complex

The CoREST complex mediates the repressive activity of the RE1 silencing transcription factor (REST) that is a key regulator of the expression of neuronal genes (Ballas et al., 2001). It comprises of histone deacetylases, HDAC1 and HDAC2 (Humphrey et al., 2001; You et al., 2001; Hakimi et al., 2002). Additionally the CoREST co-repressor complex can recruit histone methyltransferase, G9a and histone demethylase, LSD1 to reinforce repressive chromatin marks at target promoters (Lunyak et al., 2002; Roopra et al., 2004).

Table 1: Examples of co-repressors in vertebrates

Co-repressor	Transcription factor (TF)	Mechanism of activity
Metastasis associated proteins (MTA)	p53, ERα	MTA1/2 recruit histone deacetylases (HDAC), HDAC1/2 components of the NuRD co-repressor complex at targets (Manavathi et al., 2007),
BCL-6 co-repressor (BCOR) BCOR-L1	BCL-6, AF9	Recruits FBXL10 (Jmjc histone demethylase) (Wamstad et al., 2008). BCOR-L1 is related to BCOR and recruits HDACs and CtBP (Pagan et al., 2007).
MEF2 interacting transcription repressor (MITR)	MEF2 family of TFs	Interacts with CtBP and recruits HDACs (Zhang, C. L. et al., 2001)
SHARP	RB-J_K/CBF1	Interacts with HDAC1/2, MTA2, MBD3, and RbAp48 components of the NuRD complex (Oswald et al., 2002).
CBF interacting co-repressor (CIR)	CBF1	Interacts with HDAC2 and SAP30 components of the Sin3 complex (Hsieh et al., 1999).
c-Ski and *ski*-related	SMAD2,3,4 and RB	Ski recruits HDACs of the Sin3 and

Co-repressor	Transcription factor (TF)	Mechanism of activity
novel gene (*sno)*		SMRT/N-CoR complexes (Liu et al., 2001).
RING finger LIM domain binding protein (Rlim/Rnlf2)	LIM domain containing TFs	Rlim interacts with Sin3 co-repressor complex (Bach et al., 1999).
Alien	Thyroid hormone receptor (TRα/β) and vitamin D receptor (VDR)	Interacts with HDAC1/3 and Sin3 complex (Dressel et al., 1999)

1.7 MOST REPRESSORS RECRUIT MULTIPLE CO-REPRESSORS

Theoretically, the recruitment of a single co-repressor could be sufficient for a repressor to silence all of its target genes. However, many repressors can recruit more than one co-repressor; for example, in *Drosophila* the repressors Hairy, Hairless, Knirps and Brinker (Brk) can each recruit two co-repressors, Gro and CtBP via conserved short, 4-10 amino acid recruitment motifs – CiMs and GiMs (CtBP- and Gro-interaction motifs) (Paroush et al., 1994; Nibu et al., 1998a; Poortinga et al., 1998; Hasson et al., 2001; Zhang, H. et al., 2001; Barolo et al., 2002; Payankaulam and Arnosti, 2009). As mentioned above CtBP activity is limited to short distances

of up to 100 bp from a repressor binding site (Nibu et al., 1998a; Chinnadurai, 2007) [see sub-section 1.5.1], while Gro has been shown to function over much longer range, at least when recruited by some repressors such as Hairy (Barolo and Levine, 1997; Martinez and Arnosti, 2008) [see sub-section 1.5.2], although when recruited by Knirps it has similar short-range properties to CtBP (Payankaulam and Arnosti, 2009). Consequently, it is unclear what CtBP can do that Gro cannot and poses the question: why is Gro alone not sufficient?

Possible reasons are as follows. (i) Quantitative: two co-repressors may additively provide more repressive activity than can be provided by one alone. (ii) Qualitative: one co-repressor may provide a unique activity not provided by the other and which is essential for repression of one or more target genes. Alternatively, a transcription factor may be unable to recruit one co-repressor at some targets where the other would be required. (iii) To minimize noise: a second co-repressor may serve as a backup to ensure the transcription factor works efficiently all the time. This is distinct from the 'quantitative' possibility, because in that scenario one co-repressor would never be sufficient alone to repress one or more targets, while 'noise reduction' suggests that one co-repressor would be sufficient almost all of the time, but occasionally not. (iv) Availability: each co-repressor may not be expressed or active in all cells in which the transcription factor functions.

Both CtBP and Gro appear to be expressed ubiquitously (Delidakis et al., 1991; Nibu et al., 1998b; Poortinga et al., 1998; Jennings and Ish-Horowicz, 2008) but Gro activity can be downregulated by phosphorylation downstream of Receptor Tyrosine Kinase (RTK) signaling cascades (Hasson et al., 2005; Cinnamon et al., 2008).

1.7.1 Studies on transcription factors Hairy, Hairless and Knirps

Previous studies on the transcription factors Hairy, Hairless and Knirps have tried to address these possibilities genetically and determine why these repressors recruit both CtBP and Gro. First by determining if the repression of known target genes of these factors is dependent upon Gro or CtBP by assessing mutants of each co-repressor.

In *gro* null mutants embryos the striped pattern of expression of the Hairy target gene, *fushi-tarazu* (Howard and Ingham, 1986) is completely disrupted and merged (Paroush et al., 1994; Jennings et al., 2008). While Hairy targets like *string* and *kayak* were derepressed in *CtBP* but not in *gro* mutant embryos suggesting that they rely on CtBP alone for their regulation (Bianchi-Frias et al., 2004).

In the early *Drosophila* embryo the pair rule gene *even-skipped* is expressed in a seven stripe pattern and Knirps is required to limit the width of expression of some of these stripes which expand in Knirps mutants. Some of these stripes also expand in CtBP mutants indicating Knirps needs to recruit CtBP to regulate the expression of these stripes (Struffi et al., 2004). However loss of *knirps* is more severe than loss of *CtBP* suggesting that Knirps utilizes CtBP independent mechanisms as well for repression (Keller et al., 2000; Struffi et al., 2004) and this is largely attributed to Gro, but the complete loss of maternal Gro results in pleiotropic effects on the *even-skipped* phenotype precluding its analysis in *gro* null mutants (Payankaulam and Arnosti, 2009).

Using RNAi, Gro was shown to be essential for repression of the Hairless target, vg^{QE} (an enhancer of the *vestigial* gene), but using a similar approach, no evidence for CtBP requirement was uncovered (Nagel et al., 2005).

The second genetic approach was to analyze repression of target genes following

overexpression of the wild-type transcription factor and its mutant forms that lack the ability to recruit CtBP or Gro. In general, proteins that lack the CiM or GiM are less active than wild-type when overexpressed, although it is not always clear if protein levels are comparable between the mutants and wild-type. The misexpression of mutant Hairy protein unable to recruit CtBP results in the strong derepression of its embryonic target genes *tailless, forkhead* and *huckebein,* in contrast there is only a minor effect on their expression following the overexpression of a mutant form of Hairy protein that is unable to recruit Gro suggesting that Gro is its primary co-repressor (Zhang and Levine, 1999). Misexpression based analyses with mutant forms of Hairless in different stages of eye development reveals that Gro and CtBP may be acting in concert in the specification of photoreceptor cells but they are differentially utilized during the proliferative and apoptotic phases of eye development (Nagel et al., 2005; Nagel and Preiss, 2011). Analyses with mutant forms of Knirps unable to recruit CtBP or Gro suggests that together they provide higher repressive effects than either acting alone and further the N-terminally truncated version of Knirps that recruits Gro alone, when expressed at sufficiently high levels can even efficiently repress Knirps targets dependent on its CtBP mediated activity as revealed by loss of function studies (Struffi et al., 2004; Payankaulam and Arnosti, 2009).

Further analyses based on a chromatin profiling approach, DamID in both cell culture and in embryos revealed that Hairy appears to only recruit Gro to targets while it recruits only CtBP to others (Bianchi-Frias et al., 2004). Also *in vitro* cell culture assays showed that mutant forms of Hairless unable to recruit Gro and/or CtBP show markedly reduced repression compared to the wild-type, indicating that Gro and CtBP maybe acting in a combinatorial fashion to facilitate Hairless mediated repression (Nagel et al., 2005).

These studies do not provide strong conclusions. For Hairy, Gro appears to be the

primary co-repressor and the loss of its ability to recruit CtBP is less detrimental when compared to the activity of mutant form of Hairy that is unable to recruit Gro (Zhang and Levine, 1999). In contrast, DamID profiling in cells and embryos suggest that a majority of Hairy targets are associated with CtBP and only a small subset are associated with Gro (Bianchi-Frias et al., 2004). For Hairless, Gro and CtBP appear to be acting in concert in some contexts while in others they appear to be utilized differentially and this is not well understood (Nagel et al., 2005; Nagel and Preiss, 2011). And finally for Knirps the full-length protein has a greater repressive activity compared to mutant forms that can recruit either Gro or CtBP but it is unclear still as to why Knirps recruits both (Struffi et al., 2004; Payankaulam and Arnosti, 2009).

Thus, for all transcription factors that recruit both Gro and CtBP previous analyses have uncovered that some targets appear to be dependent on CtBP while for others Gro maybe required, while many targets can be repressed if only one is available, in particular if the repressor is overexpressed. The reason for these differences is not understood.

1.8 BRINKER IS A SEQUENCE SPECIFIC TRANSCRIPTIONAL REPRESSOR

Brinker (Brk) is a 704 amino acid transcription factor, containing a helix-turn-helix (HTH) DNA binding domain, interaction motifs for the co-repressors, CtBP and Grouch (Gro), CiM and GiM respectively and an additional 3R repression domain (Fig. 6A) (Winter and Campbell, 2004). The presence of the DNA binding domain at the N-terminus of Brk was confirmed by electrophoretic mobility shift assay (EMSA) and further using DNase I footprinting assays Brk was shown to bind the GGCGYY (where Y is any pyrimidine residue) consensus sequence at target genes (Sivasankaran et al., 2000). The Brk DNA binding domain shows a weak homology

to homeodomains with only three out of 13 amino acid residues that form 70% consensus sequence of the homeodomain family conserved in Brk (Cordier et al., 2006). In addition the GC-rich Brk binding sequence differs from the AT-rich recognition sequence of homeodomain proteins (Sivasankaran et al., 2000). The Brk DNA binding domain comprises of four α helices containing a HTH DNA recognition motif identified by NMR spectroscopy; the recognition helix α3 mediates DNA contacts and confers specificity while the N-terminal arm and beginning of helix α2 mediates non-specific contacts to the DNA backbone (Cordier et al., 2006). Further Brk DNA binding domain contains two α helices N-terminal to the HTH motif, in contrast to homeodomains that contain only a single one (Cordier et al., 2006). While HTH motif containing proteins, for example the lambda repressor always bind DNA as a dimer, homeodomains can bind DNA as monomers although in some cases they can also bind as dimers (Beamer and Pabo, 1992; Merabet and Ackers, 1995; Birrane et al., 2009; Kato et al., 2010). NMR analysis reveals that Brk binds DNA as a monomer and currently there is no evidence to suggest that it may bind DNA as a dimer but further analysis is required to completely rule out the latter (Cordier et al., 2006). Further this analysis indicated that Brk DNA binding domain is completely unfolded in solution but upon binding DNA it transitions to a completely folded structure (Cordier et al., 2006).

The ancestral Brk protein from Hemipterans (aphids), Coleopterans (beetles) and Hymenopterans (ants and wasps), identified by presence of DNA binding domains very similar to that from *Drosophila melanogaster* possess two DNA binding domains. The reduction to a single domain appears to have occurred at least twice, in Lepidopterans (butterflies and moths) and in Dipterans, the latter occurring fairly recently as the vestiges of the second domain can be identified in mosquitoes, but not in *Drosophila* species. One possibility is that in the ancestral

condition the protein binds two non-adjacent sites using its separate DNA binding domains and, if true, this may be replicated in a species with Brk containing a single domain if it can dimerize. It should be noted that so far all the structural analysis has been performed with only the N-terminal fragment of Brk (Cordier et al., 2006), so the question of whether Brk binds DNA as a monomer or dimer needs to be re-addressed with full-length protein.

Brk regulates patterning of various ectodermal tissues in different stages of *Drosophila* development and an overview of Brk expression and activity in various tissues has been shown in Fig. 6.

1.8.1 Brinker function in embryogenesis

Brinker (Brk) expression is initiated ventrolaterally in the late syncytial to early cellular blastoderm embryo (Fig. 6C). It is activated at least in part by the maternal Dorsal protein and repressed later in embryogenesis by Dpp signaling (Jazwinska et al., 1999a). It aids in dorsoventral patterning of the embryo by antagonizing and restricting the expression of *decapentaplegic* (*dpp), zerknullt (zen), schnurri (shn), crossveinless-2 (cv2)* and *tolloid (tld)* to the dorsal regions of the embryo where they are required for proper patterning and producing the dorsal ectoderm and amnioserosa (Ip et al., 1991; Huang et al., 1993; Kirov et al., 1994). Thus, in *brk* mutants these dorsally expressed *brk* targets are derepressed and expand ventrally (Fig. 6G,J). Brk has been shown to directly bind to enhancers of some of these embryonic targets (Rushlow et al., 2001).

Later in embryogenesis Brk is expressed ubiquitously in the ventral ectoderm (Fig. 6D) and is required to establish the characteristic pattern of ventral abdominal denticle belts of the larva; denticles are hair-like protrusions of the larval cuticle (Lammel et al., 2000; Saller et al.,

2002). In the wild-type ventral ectoderm each abdominal segment contains a denticle belt located in the anterior. Each ventral denticle belt (VDB) comprises of six rows of denticles that exhibit a distinct polarity, thus those in rows 1 and 4 point anteriorly while the rest point posteriorly (Fig. 6H) (Saller and Bienz, 2001). Consistent with Brk function in VDB patterning, in *brk* null mutants VDBs are significantly reduced with loss of most denticles while those that remain lose polarity and point posteriorly (Fig. 6K) (Saller and Bienz, 2001).

1.8.2 Brinker function in the wing disc

1.8.2.1 Wing imaginal disc and the adult wing

In *Drosophila* the precursors of adult appendages – wing, haltere and leg originate in the embryo as mono-layered sac-like clusters of polarized, columnar epithelial cells known as imaginal discs (Cohen, 1993). The wing disc is divided into precise anterior (A) and posterior (P) compartments along the A/P axis and the dorsal (D) and ventral (V) compartments along the D/V axis and there is no intermingling of cells between the compartments, as the compartment boundaries are strict lineage restrictions (Fig. 7A) (Bryant, 1970; Garcia-Bellido et al., 1973; Blair, 1995). Upon metamorphosis, as the wing disc everts, its ventral surface folds under the dorsal surface in the distal region to produce the adult wing, the boundary of the dorsal and ventral compartments produces the wing margin, the anterior and posterior compartment boundary however corresponds to the center of the wing between wing veins III and IV and the wing pouch, the central region of the disc forms the wing blade (Fig. 7B) (Cohen, 1993).

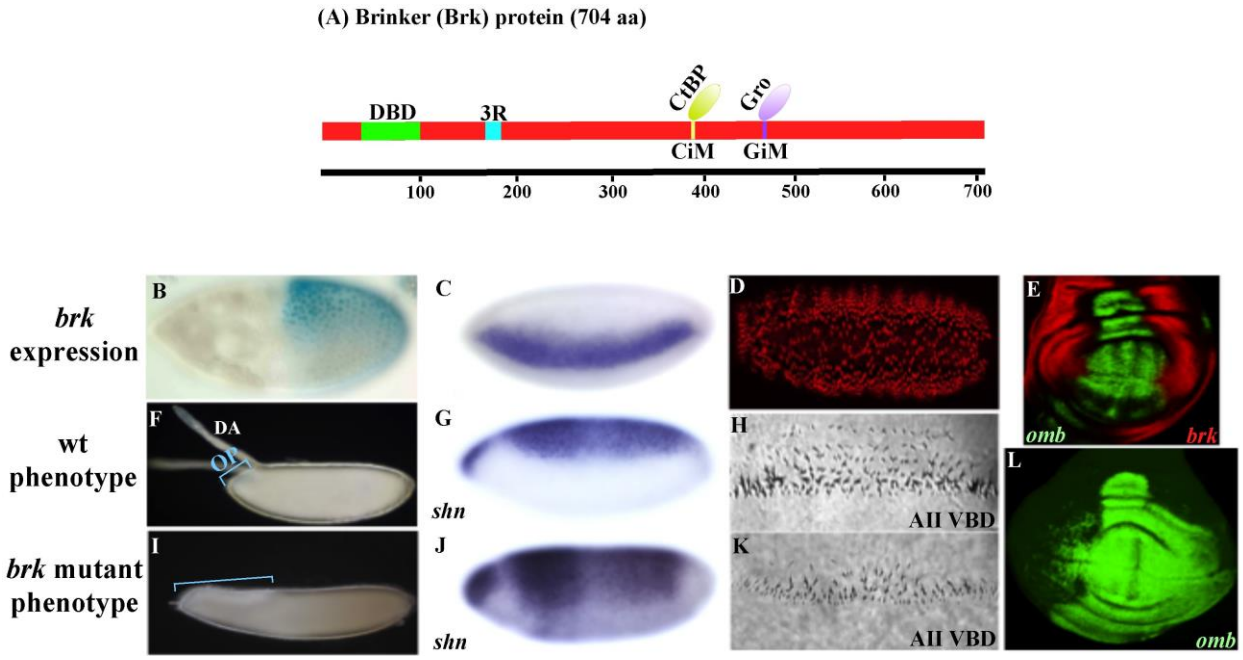

Figure 6: Overview of Brinker (Brk) protein

(A) The *brk* gene encodes a 704 amino acid protein with helix-turn-helix DNA binding domain (DBD), interaction motifs to recruit the co-repressors CtBP and Grouch (Gro) or CiM and GiM respectively and a histidine and alanine rich additional repression domain, 3R. (B-E) Wild-type Brk expression. All images anterior left, dorsal up except H,K. (B) During oogenesis, Brk (lacZ) is expressed in the follicle cells associated with the oocyte. (C) In the early, cellular blastoderm embryo Brk is expressed ventrolaterally. (D) In the late embryo it is expressed ubiquitously across the ventral ectoderm (lacZ; red). (E) In the larval wing disc Brk is expressed in a lateral to medial gradient (αBrk, red) and its target *omb* (lacZ; green) is medially expressed. (F) Wild-type phenotype of egg-shell structures - the dorsal appendages (DA) and operculum (OP) secreted by follicle cells. (G) Wild-type expression of the Brk target *shn* in blastoderm embryo. (H,K) Anterior top. 2nd abdominal (AII) ventral denticle belt (VDB). (H) Wild-type AII VDB, denticles in rows 1 and 4 point anteriorly while the rest point posteriorly. (I-L) *brk* mutant phenotype. (I) In eggs obtained from mothers with *brk* null mutant clones in follicle cells DAs are lost and OP is expanded significantly. (J) Brk antagonizes dorsally expressed genes like *shn* that are derepressed in *brk* null mutant embryos. (K) Later embryogenesis: *brk* null mutants possess narrowed AII VDB with most denticles lost; all remaining denticles lose polarity and point posteriorly. (L) In *brk* mutant wing discs the *brk* targets like *omb* are derepressed and their expression extends to the edge of the wing disc.

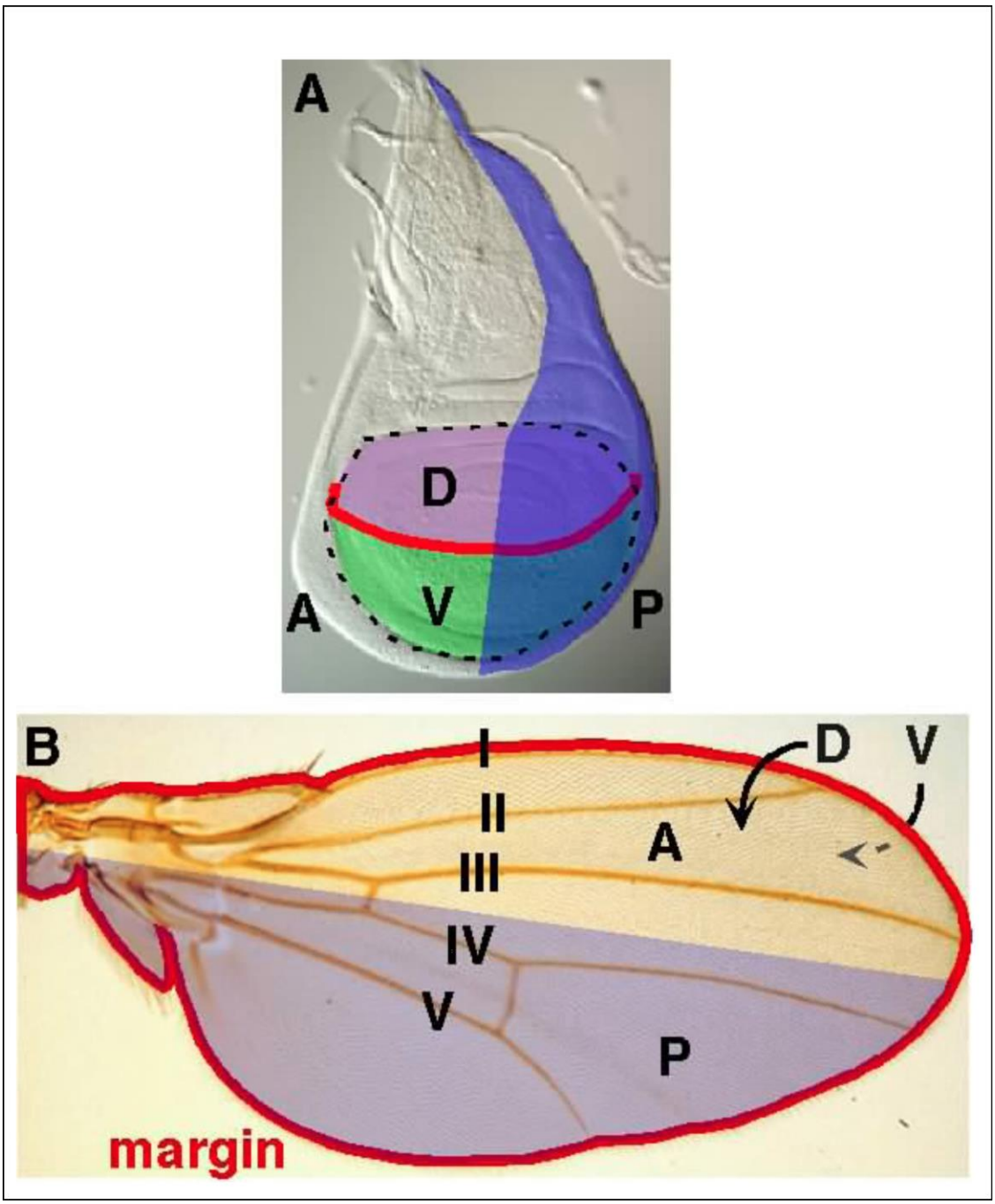

Figure 7: Fate-map of the wing imaginal disc and its derivatives in the adult wing.

(A) Fate-map of the wing imaginal disc depicting regions that give rise to structures in the adult wing. Only the central portion (dashed region) of the wing disc, the wing pouch gives rise to the wing blade. The wing disc is divided into the dorsal (D), ventral (V), anterior (A) and posterior (P) compartments. (B) The ventral surface of the wing disc folds under the dorsal surface and fuses to form the wing. The wing veins I, II, III, IV and V have been indicated and the regions of the wing disc that give rise to corresponding areas and structures of the adult wing have been color coded.

1.8.2.2 Brinker is a negative regulator of Dpp 'targets' in wing patterning

The *Drosophila* wing patterning along the A/P axis and D/V axis is brought about by the gradient of the morphogens, Decapentaplegic (Dpp) and Wingless (Wg), respectively (Schwank and Basler, 2010). Dpp is a member of the TGF-β superfamily of signaling molecules and is closely related to the vertebrate Bone Morphogenetic Proteins (BMPs). It is expressed in a narrow stripe of cells just anterior to the anterior and posterior compartment boundary in the developing wing disc, it spreads largely by facilitated diffusion away from its source to generate symmetrical medial to lateral concentration gradients along the A/P axis and thereby directly exerting long range organizing effects on cells in the wing disc (Fig. 8) (Lecuit et al., 1996; Nellen et al., 1996; Affolter and Basler, 2007).

Just like other TGF-β family members, the extracellular Dpp ligand binds the type I transmembrane serine-threonine kinase receptors, Thickveins (Tkv) or Saxophone (Sax), that in turn binds the type II serine-threonine kinase receptor, Punt to form a heteromeric receptor complex (Brummel et al., 1994; Letsou et al., 1995; Ruberte et al., 1995; Penton and Hoffmann, 1996). Punt (Put), a constitutively active kinase then phosphorylates and activates Tkv, the type I receptor in the wing, which in turn phosphorylates the Smad intracellular transducer, Mothers-Against-Dpp (Mad) (Sekelsky et al., 1995; Newfeld et al., 1996). Phosphorylated Mad (pMad) interacts with co-Smad Medea and this complex is translocated to the nucleus where it regulates transcription of target genes (Das et al., 1998; Hudson et al., 1998; Xu et al., 1998). In addition, a Dpp target Daughters-Against-Dpp (Dad), an inhibitory Smad, negatively regulates the Dpp activity gradient by competing with Mad for binding to the Tkv receptor and inhibiting its phosphorylation (Tsuneizumi et al., 1997).

Dpp signaling can directly repress target gene expression acting via pMad and Medea

which bind together with the Schnurri protein at well defined silencer elements in genes negatively regulated by Dpp. (Pyrowolakis et al., 2004). In the wing disc it directly represses Brinker (Brk), which is consequently expressed in a lateral to medial gradients, complementary to the medial to lateral Dpp gradients (Fig. 6E and 8) (Campbell and Tomlinson, 1999; Jazwinska et al., 1999b; Minami et al., 1999; Marty et al., 2000; Muller et al., 2003). Brk functions to repress expression of medially expressed wing genes, including *spalt* (*sal*) and *optomotor-blind* (*omb*). These genes were originally classified as Dpp targets, and in other tissues some Dpp target genes can be activated directly by pMad and Medea (in the absence of Schnurri), but *sal* and *omb* are regulated largely by Brk, or rather its absence – they are expressed in the medial region of the wing because Dpp eliminates Brk from here (Campbell and Tomlinson, 1999; Jazwinska et al., 1999b; Minami et al., 1999; Muller et al., 2003). Consistent with this, in *brk* mutants its targets like *sal* and *omb* are derepressed (Fig. 6L). In fact, they show similar derepression in *brk dpp* double mutants, reinforcing the view they do not need direct activation via Dpp (Campbell and Tomlinson, 1999). Sal and Omb are directly repressed by Brk binding to their enhancers (Sivasankaran et al., 2000; Barrio and de Celis, 2004).

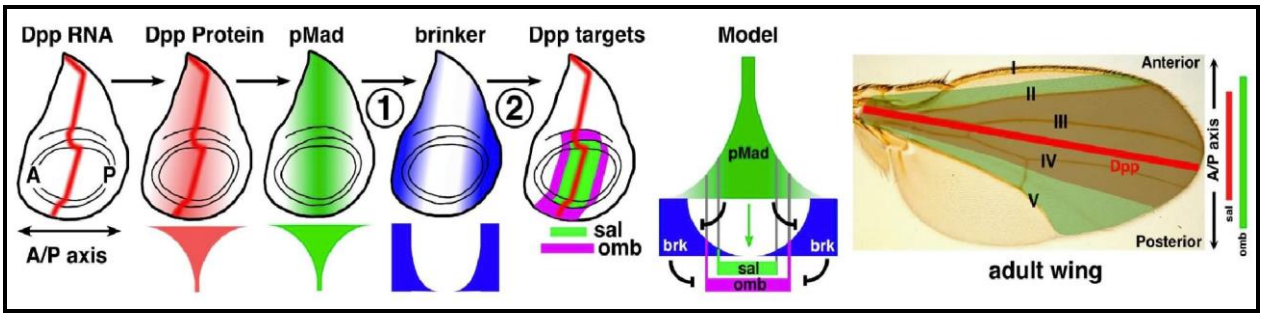

Figure 8: Regulation of gene expression in the *Drosophila* wing by Dpp and Brk.

The *dpp* mRNA is expressed in middle of the wing imaginal disc along its A/P axis, this is translated into the Dpp protein, a secreted signaling molecule that becomes distributed in a medial to lateral gradient with highest levels along the A/P axis and progressively lower levels away from it. Dpp signaling establishes a phosphorylated Mad (pMad) gradient that mirrors the Dpp gradient. Brinker (Brk), a transcriptional repressor is expressed in a lateral to medial gradient in the wing imaginal disc. Dpp signaling acting via pMad regulates its target genes *sal* and *omb* indirectly by repressing Brk and eliminating its expression from the medial region of the wing to allow their expression. Sal and Omb are differentially sensitive to Brk, such that Sal is repressed by it at low levels and is expressed in a narrower domain than Omb that requires higher Brk levels to be repressed. Also is shown the regions of the adult wing arising from specific regions in the larval wing disc.

1.8.3 Brinker function in oogenesis

1.8.3.1 Oogenesis

A female *Drosophila* has two ovaries made up of 14-18 ovarioles, each of which consists of a series of developing egg-chambers, which develop in an assembly line fashion into an egg (King, 1970; Spradling, 1993; Margolis and Spradling, 1995; Bastock and St Johnston, 2008). The germarium containing the germline and somatic stem cells is located at the anterior tip of the ovariole. The progeny of both germline and somatic stem cells give rise to egg chambers, which pinch off and are associated to the adjacent egg-chambers by stalk cells like beads on a string (Fig. 9) (He et al., 2011). Each egg-chamber matures through 14 morphologically distinct stages over close to a week traveling along the ovariole to reach the posterior as a mature egg competent for fertilization (Fig. 9) (King, 1970; Spradling, 1993). Each egg-chamber is comprised of an oocyte and its 15 sister nurse cells that together form the germ cell cyst, surrounded by around 650 somatically derived follicle cells (Becalska and Gavis, 2009). The nurse cells produce maternal factors such as mRNAs, proteins and organelles that are delivered to the oocyte and are critical for its development as the oocyte nucleus is largely transcriptionally

quiescent (Horne-Badovinac and Bilder, 2005). While the epithelial follicle cells secrete ligands or activators instrumental in establishing polarity within the oocyte and subsequently the embryo, they also synthesize and transport yolk polypeptides to the oocyte and finally produce the egg-shell layers and specializations such as the dorsal appendages, operculum and micropyle. The dorsal appendages are a pair of tubes on the dorsal-anterior that aid in respiration, the operculum is a lid-like structure through which the larva hatches and the micropyle at the anterior-most pole of the egg is required for sperm entry (Ray and Schupbach, 1996; Waring, 2000; Wu et al., 2008).

1.8.3.2 Brinker patterns *Drosophila* egg-shell structures

Brinker (Brk) expression is initiated at stage 8 of oogenesis in most of the oocyte associated follicle cells (Chen and Schupbach, 2006). It is repressed by Dpp expressed in the anterior follicle cells and is expressed in a complementary posterior to anterior gradient (Fig. 6B), in addition Brk expression is upregulated by Epidermal Growth Factor Receptor (EGFR) signaling (Chen and Schupbach, 2006). Brk helps in patterning critical egg-shell structures secreted by the follicle cells such as the dorsal appendages and the operculum. Thus, *brk* null mutant eggs have a characteristic phenotype, with a complete loss of dorsal appendages and a significant expansion of the operculum (Fig. 6F,I) (Chen and Schupbach, 2006; Shravage et al., 2007). No direct targets of Brk have been identified in the follicle cells.

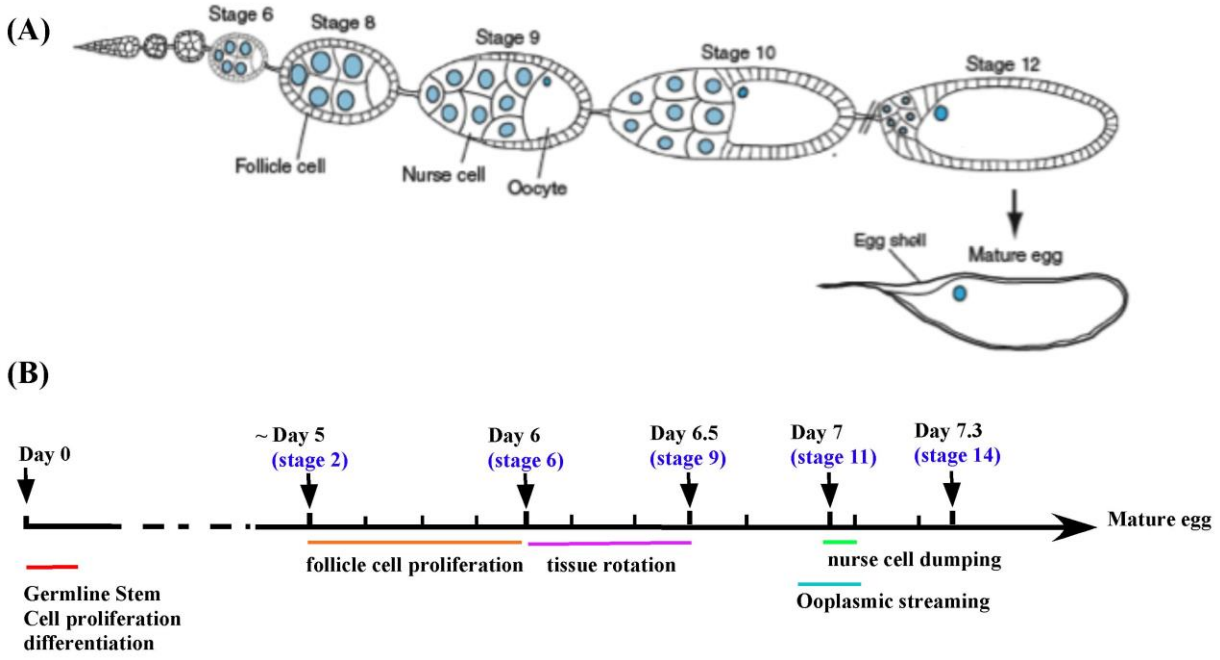

Figure 9: Overview of *Drosophila* oogenesis

(A) Each *Drosophila* ovary consists of 14-18 ovarioles, each of which in turn acts as an egg production line. Egg-chambers arise from the germarium situated at the anterior of the oocyte and progress through 14 morphologically distinct stages over about a week. Each egg-chamber is composed of an oocyte, 15 polyploid nurse cells that together form the germ cell cyst and are surrounded by the somatically derived follicular epithelium. During most of oogenesis nurse cells transfer maternal factors such as RNA and protein to the oocyte, which is largely transcriptionally quiescent. However at stage 10 when the nurse cell cluster and the oocyte become similar in volume the nurse cells extrude their contents into the oocyte, followed by vigorous streaming of the oocyte cytoplasm (ooplasmic streaming) that aids in intermixing the nurse cell cytoplasm with that of the oocyte, followed by elimination of the nurse cells. The follicle cells give rise to specializations of the egg-shell membrane and help pattern the egg-chamber. (B) Timeline of major developmental events in *Drosophila* oogenesis. The beginning of each developmental stage is indicated as a mark on the line and the interval shown between stages is proportional to the approximate development time. Adapted from (Becalska and Gavis, 2009) and (He et al., 2011)

1.9 MECHANISM OF BRINKER MEDIATED REPRESSION

Previous studies had suggested that Brk might function passively by binding site competition with activators as its binding sites overlap with that of an unknown activator in the *omb* wing enhancer (Sivasankaran et al., 2000). Further support for this came from studies in the embryos, where Brk targets *zen* and *Ultrabithorax (Ubx)* contain binding sites that overlap with activator binding sites (Mad) and *in vitro* binding assays showed that Brk and Mad compete for the same or overlapping regions of DNA (Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller and Bienz, 2001). However, subsequent studies in the wing and embryo suggested that a Brk protein containing the DNA binding domain (DBD) alone had no repressor activity and that an additional repression domain was required, indicating that at least *in vivo* Brk does not function by simple binding site competition (Winter and Campbell, 2004).

Brk possesses recruitment motifs for the co-repressors, CtBP and Gro, CtBP- and Gro-interaction motifs, CiM and GiM respectively that are evolutionarily conserved, in addition to an independent repression domain, 3R that is not conserved (Winter and Campbell, 2004). Assessment of whether either or both the co-repressors and the 3R domain are required for Brk activity was carried out in the wing disc by generating *CtBP* and *gro* single and double mutant clones. Additional analyses involved generating brk^{F124} (*brk* null mutant) and brk^{F138} (*brk* hypomorph with the 3R domain alone functional) homozygous clones in the wing disc. These studies revealed that Gro is essential for Brk activity in the wing disc and suffices for the repression of its target *sal*, although CtBP can provide some, but not complete, back up activity in its absence (Winter and Campbell, 2004). In contrast, neither Gro nor CtBP were revealed to be required for repression of another target *omb* as the 3R domain is sufficient in the absence of Gro and CtBP for its repression (Winter and Campbell, 2004).

Misexpression of a modified form of Brk that possesses only the 3R domain and is unable to recruit both Gro and CtBP in the wing disc is more effective at repressing *omb* than *sal*. In contrast, mutant Brk forms possessing either the GiM or CiM when overexpressed were found to efficiently repress both *sal* and *omb* (Winter and Campbell, 2004).

Moreover, misexpression of modified Brk proteins that are unable to recruit CtBP or Gro in the embryo suggested that for the regulation of *zen* Brk does not need either Gro or CtBP, while it requires Gro but not CtBP for repression of *tld* and requires both Gro and CtBP to efficiently repress *dpp* (Hasson et al., 2001; Zhang, H. et al., 2001).

Taken together these findings suggested that Gro is sufficient for Brk activity in the wing and largely sufficient in the early embryo leaving it unclear as to why it possesses the 3R domain or can recruit CtBP. Importantly the CiM in Brk has been evolutionarily conserved for over 300 million years (Winter and Campbell, 2004) and therefore is likely to mediate important function(s), hitherto unidentified that presumably cannot be discharged by Gro.

1.10 PROJECT GOALS

The studies described above (sub-sections 1.7 and 1.9) have not been conclusive in uncovering why transcription factors such as Hairy, Hairless, Knirps and Brinker possess recruitment motifs for both Gro and CtBP. For Brk, in the wing and the embryo, Gro appears to be essential (Hasson et al., 2001; Zhang, H. et al., 2001; Winter and Campbell, 2004) and there is no obvious reason why it needs to recruit CtBP or why it possesses the 3R domain.

The approaches utilized in these earlier studies have several drawbacks, for example: (a) Analyses have been limited to certain tissues. (b) *CtBP* and *gro* loss of function tend to have

pleiotropic effects as they are utilized by many other transcription factors. (c) Overexpression is not easily compared to wild-type function, not least because the levels produced rarely mirror that of the endogenous protein. A further problem is that several transcription factors have additional repressive activities independent of CtBP and Gro (Winter and Campbell, 2004; Nagel et al., 2005).

The most direct approach to address this issue would be to compare the activity of Brk from mutants in which the CiM and/or the GiM are nonfunctional. In the case of Brk the only endogenous mutants available were brk^{F124} (has an amino acid substitution in the DBD that abolishes activity), brk^{E427} (protein truncated after the DBD) and brk^{F138} (protein truncated before the CiM and GiM leaving the 3R intact), brk^{M68} (protein truncated in the DBD) (Lammel et al., 2000; Lammel and Saumweber, 2000). However no mutants exist carrying mutations in each repression domain and consequently I have generated a series of endogenous *brk* mutants in which the CiM, GiM and 3R are mutated individually or in combination. This was achieved using the genomic engineering approach of Hong and colleagues (Huang et al., 2009) in which a gene is replaced by an *attP* ØC31 bacteriophage integration site then allowing insertion of modified/mutated forms into this site essentially replacing the endogenous gene with these forms. I then analyzed the activity of each of these endogenous Brk mutants in the different tissues in which Brk is known to function.

These studies revealed that Gro is necessary and almost sufficient for Brk to contribute to generate a morphologically wild-type fly, but it cannot do so efficiently and repeatedly. CtBP and 3R together provide increased repression and may mediate noise reduction to supplement Gro activity and ensure that it can function consistently. Also Gro is insufficient during oogenesis where CtBP and 3R are essential. Here, Brk activity coincides with high levels of

Receptor Tyrosine Kinase (RTK) signaling that have been shown previously to downregulate Gro activity (Hasson et al., 2005; Cinnamon et al., 2008), making it unavailable for Brk and therefore explaining why Brk requires additional mechanisms for repression. The findings of the present study provide important insights into why transcription factors in general may recruit more than one co-repressor and is the first to dissect the mechanism of activity of a transcription factor in a multicellular eukaryote in a physiologically relevant manner.

2.0 GENERATION AND VALIDATION OF BRINKER MUTANTS

2.1 INTRODUCTION

In the current study my goal was to generate a series of endogenous Brinker (Brk) mutants in which the CtBP interaction motif (CiM), Groucho interaction motif (GiM) and 3R were mutated individually and in combination. Here I will initially review the methods available to generate mutations in flies and explain why I am using the genomic engineering approach of Hong and colleagues (Huang et al., 2009) that is a modification of the homologous recombination based 'ends out' gene targeting approach introduced by Golic and colleagues (Gong and Golic, 2003). Using this gene targeting approach I generated a *brk* knockout, brk^{KO} in which the endogenous *brk* gene was replaced by ØC31 bacteriophage *attP* site. This was then used to integrate modified forms of *brk* where the CtBP interaction motif (CiM), Groucho interaction motif (GiM) and 3R were mutated individually and in combination to create mutants where the native *brk* gene is essentially replaced by the mutant forms, these were then used to dissect Brk activity in all tissues where Brk is known to function.

2.1.1 Forward genetics in *Drosophila*

Mutations have been uncovered in genes in *Drosophila melanogaster* for over a hundred years. Methods to generate these mutations include the following. (a) Random causes. Initially workers just pushed flies around hoping for a spontaneous mutation to arise. Subsequent analysis many years later revealed the majority of these were associated with the insertion of a transposable element. (b) Transposable elements. In particular, the P-element (Rubin and Spradling, 1982) has been used to generate mutations through insertion into genes and regulatory regions. P-elements also have the advantage that the mobilization of an element can occasionally result in deletion of adjacent DNA. Its use as a mutagenic agent is reduced somewhat by the fact that it does not insert completely randomly into the genome. This has led to the use of other transposons, in particular the piggyBac element, although this has the disadvantage of not producing deletions upon mobilization (Thibault et al., 2004). (c) Radiation, in particular X-ray and γ-ray. It became obvious fairly early on that these mutations were usually associated with chromosomal aberrations such as deletions and inversions. (d) DNA damaging chemicals, in particular ethyl methanesulfonate (EMS). These usually cause point mutations, but also cause a high percentage of small deletions.

For my project I wanted to generate mutations that disrupted specific regions of the *brk* gene – the CiM, GiM and 3R - and so the question to be addressed is whether any of these forward genetic approaches might meet this goal. In general, transposon and radiation mutagenesis would not generate these types of mutations as these usually just result in a simple break in a gene. Chemical mutagenesis does have the potential to generate such mutations, but there are two problems. First, to identify the desired mutations they should either be lethal or have a specific loss of function phenotype over existing *brk* mutants and it was not clear if these

mutations would have this property. In hindsight, an F2 EMS screen may have generated the mutation in the GiM as in brk^{GM}, but it is unlikely that the brk^{CM} and $brk^{\Delta 3R}$ mutations would have been uncovered by this technique. Second, it would have been impossible to generate double mutants by this approach. This led me to explore alternative possibilities, in particular reverse genetics that would allow direct modifications of a gene in its endogenous location in the genome.

2.1.2 Reverse genetic approaches in *Drosophila* and other organisms

Gene targeting is the introduction of genetic modification at a genomic locus by recombination between an introduced DNA fragment and the homologous target gene. To succeed in homologous recombination it is necessary to introduce into the nucleus a linear DNA molecule with regions of 5' and 3' genomic homology between which an *in vitro* modification is to be introduced. Such gene targeting approaches for the modification of target loci has been routinely used in yeast (Rothstein, 1991) because introduction of DNA into this single celled organism is much more easily achieved than into an embryo and because embryos are limited in number. This approach, however, has been adapted for use in mice where the targeted modification is selected in embryonic stem cells in culture, circumventing both the size and number issue, these cells can subsequently be incorporated into the germ-line by injection into embryos at the blastocyst stage (Thomas et al., 1986; Doetschman et al., 1988). However, there is no equivalent of embryonic stem cells in flies, although the technology could have been applied to the pole cells (which generate the germline) if these could have been cultured *in vitro*, but that has not been successful. This has *necessitated the introduction of novel approaches in flies to get around this problem.*

The two major strategies for *in vivo* gene-targeting first introduced in *Drosophila* were the 'ends-in' or insertional targeting (Rong and Golic, 2000) and 'ends-out' or replacement targeting (Gong and Golic, 2003) approaches that both involve the introduction of the targeting cassette by transgenesis followed by *in vivo* homologous recombination and screening for the desired targeted integration. However, both strategies were limited by being extremely labor-intensive due to low frequencies of targeting and the unpredictability of the success of targeting events, which somewhat reduces the advantage they offer in terms of specificity in targeted mutagenesis (O'Keefe et al., 2007; Crown and Sekelsky, 2013). Several modifications have been introduced to both 'ends out' and 'ends in' gene targeting approaches to overcome their limitations and this includes the genomic engineering strategy discussed below.

2.1.3 Improvements to knockout technology in *Drosophila* and Genomic engineering

The 'ends out' gene targeting approach (Gong and Golic, 2003) involves the introduction of a 'donor' element containing the gene targeting cassette through P-element based transgenesis into the genome. This is followed by an ingenious generation of a linearized targeting DNA *in vivo* by using sequence specific FLP recombinase to excise the integrated 'donor' as an episomal circle that then is linearized by the rare restriction enzyme I-SceI. While this method is extremely powerful it is time and labor-intensive, also the rates of insertion with homologous recombination can vary greatly and could prove to be particularly challenging for constructs with low targeting efficiency (Manoli et al., 2005; Radford et al., 2005; Jones et al., 2007).

Hong and colleagues introduced several improvements to the 'ends out' strategy including modifications of the strains used in the targeting cross that eliminates the necessity of scaling it up (Huang et al., 2008). The latter is required for genes resistant to or with low

efficiency of homologous recombination and that involve screening of large numbers of putative candidates obtained from the targeting cross in order to identify true integration events (Larsson et al., 2004). In addition, a negative selection module that comprised of UAS-Reaper was introduced, that would be incorporated into false positive transgenics that have undergone non-targeted gene integrations (Huang et al., 2008). Reaper (Rpr) is a cell-death gene, thus driving its expression ubiquitously eliminates any false positives. To further aid the screening process a w^+ and neomycin-resistance gene cassette was introduced that provides positive selection and aids in the identification of the true gene integration events (Zhou et al., 2012).

However, even with these improvements, structure/function analyses would involve repeated rounds of this procedure and given the still low frequency of success would be unlikely to be contemplated. As an ingenious modification, Hong and colleagues combined the modified 'ends out' gene targeting approach with the bacteriophage ØC31 integrase mediated transgenesis that mediates efficient integration of the desired DNA into a target locus (Huang et al., 2009). This approach replaces a gene with a ØC31 bacteriophage *attP* site, essentially generating an *attP* knockout, into which modified genes can be introduced using a vector with *attB* sites. ØC31 integrase catalyzes uni-directional site-specific recombination between the bacteriophage ØC31 *attP* and *attB* sites and has been adapted successfully for transgenesis in *Drosophila* (Groth et al., 2004; Bischof et al., 2007). Further, unlike homologous recombination based strategies where integration efficiency may vary for different DNA substrates used, ØC31 integrase mediated transgenesis facilitates high throughput integration at target loci (Groth et al., 2000). In addition, since recombination at *attB-attP* sites generate *attL* and *attR* hybrid sites, with the *attR* site remaining at the targeted locus, Huang *et. al.* designed minimal *attP* (50 bp) and *attB* (53 bp) sites (Groth et al., 2000) respectively that can mediate efficient transgenesis in *Drosophila* and

leave only a minimal hybrid *attR* site at the final engineered locus, allaying concerns that this *attR* sequence could interfere with target gene expression in the final allele (Huang et al., 2009).

Huang *et. al.* (2009) were very successful in generating *attP* knockouts of 6 different genes and by integrating modified genes into these created more than 70 unique alleles at these loci (Huang et al., 2009). Being fortunate to have the Hong lab in close proximity I considered genomic engineering to be a good approach to attempt to generate *brk* mutants in which its repression domains/motifs, 3R, CiM and GiM were mutated individually and in all double and the triple combinations.

2.2 EXPERIMENTAL APPROACH

2.2.1 Generation of brk^{KO} mutant

To generate the brk^{KO} strain I utilized a modified 'ends out' gene targeting strategy, an outline of which has been depicted in Fig. 10A and a schematic overview of the crosses has been shown in Fig. 11. First I amplified by PCR the genomic fragments flanking the *brk* coding region extending into the 5' and 3' UTR and cloned them into the P-element vector, pGX-PCM1 (identical to pGX-*attP*-WN with the absence of the SphI site; (Zhou et al., 2012). This results in a targeting construct that positions a ØC31 *attP* site and *white-neo* marker flanked by *loxP* sites between the genomic fragments and has *Flippase Recognition Target* (*FRT*) sites, a recognition site for the rare restriction enzyme I-SceI and the UAS-Rpr negative selection module outside.

This initial targeting construct was then introduced into the germ-line of $white^{-}$ flies (*white* mutant and hence having white eyes) by random P-element mediated transformation.

Transformants were expected to carry the *white (w+)* marker and hence have red eyes; they were therefore selected on the basis of eye color. The chromosome at which the insertion had taken place within the different transformants was determined and those that had the insertion on the second or third chromosome were retained for homologous recombination, as *brk* is on the X.

Three selected transformant lines were balanced and the presence of functional *FRT* and *loxP* sites was determined in each of them by separately crossing them to *hs-Flp* and *hs-Cre* lines and evaluating the eye color variegation of the cross progeny following heat-shock. In particular the presence of the *FRT* sites indicated the ability to Flp out or excise the integrated targeting construct that will be required subsequently for the targeting cross. All three donor transformant lines exhibited similar and high levels of efficiency of Flping out the integrated targeting construct, and so one of the donor lines that had the insertion on the third chromosome was arbitrarily selected to perform the next step, that is the targeting cross. A homozygous stock was derived from this selected donor line to enhance the efficacy of the targeting cross.

In the targeting cross the chosen donor line was crossed to a line carrying both *hs-Flp* and *hs I-SceI* (Fig. 10A and 11). Progeny from this cross were heat-shocked to induce expression of Flp, which excises the donor to generate an episomal targeting construct. Heat-shock also induced the expression of the rare restriction enzyme I-SceI, its corresponding recognition sites are absent from the *Drosophila* genome and hence inducing its expression via heat-shock generates a linearized targeting construct, which provides a recombinogenic template. The *hs-Flp*, *hs-I-SceI* lines also had their Y and balancer chromosomes replaced by ones that contain *hs-hid* transgenes. Hence, the ubiquitous expression of cell-death gene *hid* is also induced by heat-shock causing lethality and eliminates all male progeny carrying the *hs-hid* chromosomes. As a result only female progeny of potentially the correct genotype *P(donor)/hs-Flp,hs-I SceI* are

expected to survive.

As a result of homologous recombination it is expected that the *white-neo* positive selection cassette and *attP* will be integrated in place of endogenous *brk*. However, the frequency of homologous recombination may vary from one DNA substrate to another and it may generate false positives, in particular ones in which the whole donor template integrates randomly. Progeny that have undergone precise homologous recombination will carry the *white-neo* cassette and hence will be resistant to the antibiotic G418, however it was not necessary to use this positive selection strategy in the current study as the initial screen against false positives using the negative selection module UAS-Rpr built at the 3' end of the targeting donor construct was very successful. The ectopic expression of the cell-death gene *reaper (rpr)* causes lethality and once the donor DNA fragment is recombined at the target gene, UAS-Rpr is lost due to homologous recombination. In contrast, non-targeted integrations will likely retain the donor DNA fragment along with the UAS-Rpr, these flies will then will be dead when the expression of Rpr is induced ubiquitously by crossing them with driver Gal4-221[*w*-] (Fig. 11). I crossed progeny obtained from the targeting cross to a line ubiquitously expressing Gal4 and screened 15,000 initial candidates for surviving female progeny carrying the *white* marker (putative $brk^{KO\text{-}w+}$ candidates). I obtained six hundred candidates, which were then tested by crossing them individually to a $white^{-}$ line. I screened the progeny obtained from each female, discarding those which produced male progeny carrying the *white* marker (discerned by their red eyes) and selecting those that produced only females carrying the *white* marker (Fig. 11). This was done because *brk* is essential for viability and is situated on the X-chromosome and hence any females that when crossed to $white^{-}$ produced males carrying the *white* marker could not be true *brk* knockouts (KO). From this screening step I identified ten putative candidate $brk^{KO\text{-}w+}$ flies. All

ten putative candidate *brk*$^{KO\text{-}w^+}$ lines were verified genetically as described below [see sub-subsection 2.2.1.1]. Five of these *brk*$^{KO\text{-}w^+}$ lines were then validated molecularly [see sub-section 2.2.1.2] and balanced stocks were set up (Fig. 11).

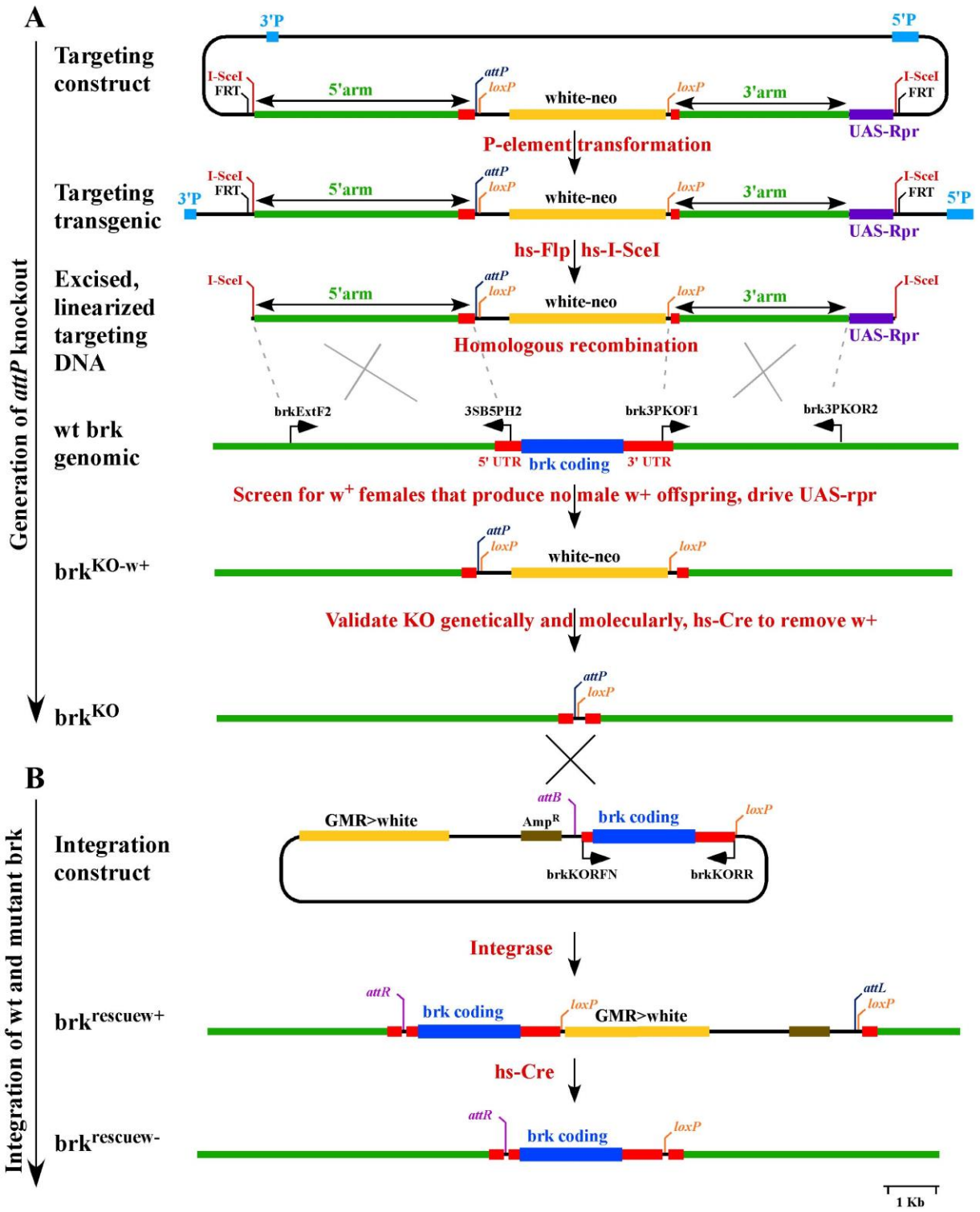

Figure 10: Generation of endogenous *brk* mutants by genomic engineering.

(A) Generation of *brk* knockout (brk^{KO}) by a modified ends out gene targeting approach. A targeting construct was made comprising: (i) 5' and 3' *brk* flanking regions/arms extending into the 5' and 3' UTRs, (ii) a ØC31 bacteriophage *attP* site positioned 3' to the 5' *brk* arm, (iii) *white* (*w+*) marker flanked by *loxP* sites, positioned between the *brk* arms (iv) UAS-rpr outside of the region containing the arms, to select against non-targeted events, (v) FRT and I-Sce-I sites that flank elements i-iv, and (vi) P-element ends for integration into the genome of *w-* flies. Following P-element mediated-transgenesis, Flippase (Flp) and I-Sce-I were used to excise and linearize targeting DNA in vivo. Non-targeted events were selected against using a ubiquitous Gal4 line to drive UAS-rpr (which will make such events lethal). Targeted events were selected for using the *w+* marker and by the fact that a *brk* KO will be on the X and will be lethal. Potential KOs were validated molecularly and genetically and finally the *w+* marker was excised using Cre, resulting in the final brk^{KO} in which the *brk* gene is replaced by an *attP* and *loxP* site.
(B) Integration of wild-type and mutant forms of *brk* into the *attP* site of brk^{KO}. Integration constructs were made consisting of a *brk* gene extending from the regions in the 5' and 3' UTRs not included in the arms used in the targeting construct, the *brk* gene is flanked 5' by an *attB* site and 3' by a *loxP* site, and a *w+* marker. This is integrated into brk^{KO} using ØC31 integrase and the *w+* marker is excised from the resulting transformants using Cre resulting in a fly carrying a *brk* gene that is identical to the wild-type with the exception of an *attR* site and *loxP* site in the 5' and 3' UTRs, respectively, along with any modification made to *brk*.

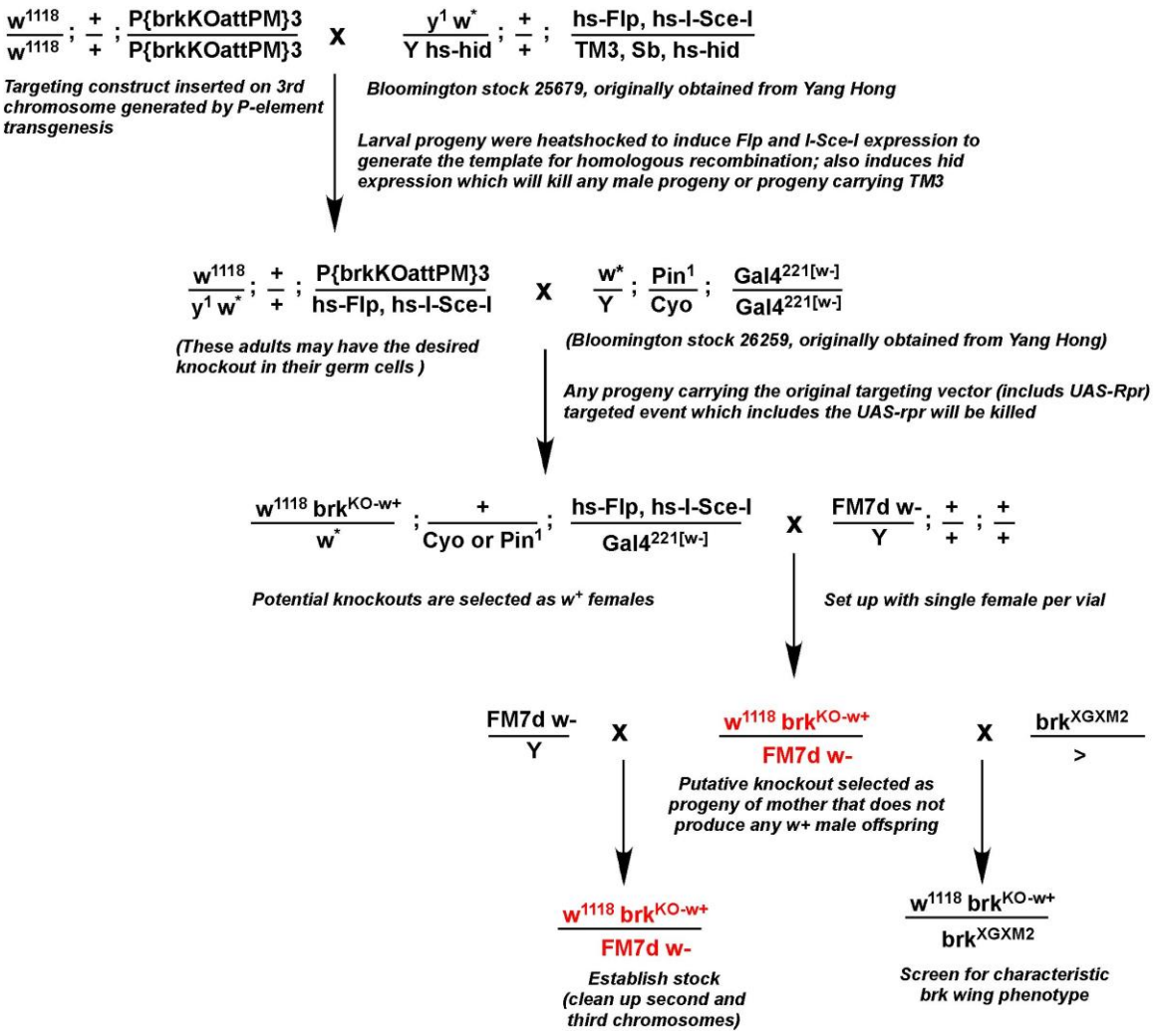

Figure 11: Schematic overview of crosses for generation of *brk^{KO}*

For the targeting cross, a homozygous line carrying the donor construct was crossed to a line carrying *hs-Flp, hs-I-SceI* and with its their Y and balancer chromosomes replaced by ones that contain *hs-hid* transgenes. Progeny from this cross were heat-shocked to induce expression of Flp, which excises the donor to generate an episomal targeting construct. Heat-shock also induced the expression of the rare restriction enzyme I-Sce-I, its corresponding recognition sites are absent from the *Drosophila* genome and hence inducing its expression via heat-shock generates a linearized targeting construct, which provides a recombinogenic template. Also heat-shock induces ubiquitous expression of cell-death gene *hid* causing strong lethality and eliminating all male progeny carrying the *hs-hid* chromosomes. As a result only female progeny of potentially the correct genotype *P(donor)/hs-Flp,hs-I SceI* are expected to survive. Putative *brk* knockout (KO) candidates were screened by crossing to a Gal4$^{221[w-]}$ that drives ubiquitous expressing of Reaper, a cell death gene in the false positives with non-targeted gene integration. Putative *brk* KO candidates were selected as progeny of mother that does not produce any w^+ males (See text, sub-section 2.2.1 for details). These were verified by genetic complementation assay by crossing to *brk^{XGXM2}* , a *brk* hypomorph and assess wing phenotype of *brk^{KO-w+}/brk^{XGXM2}*. Balanced lines were then generated with verified *brk^{KO-w+}* lines.

2.2.1.1 Genetic validation of $brk^{KO\text{-}w+}$

Putative $brk^{KO\text{-}w+}$ candidates were confirmed to be *brk* knockout flies, by a complementation test using a *brk* hypomorph line, brk^{XGXM2} (Fig. 12). The resultant $brk^{KO\text{-}w+}/brk^{XGXM2}$ mutant wing phenotype was posteriorly enlarged with an incompletely patterned 5th longitudinal (L5) wing vein (Fig. 12D). This $brk^{KO\text{-}w+}/brk^{XGXM2}$ mutant wing phenotype was indistinguishable from that of brk^{F124}/brk^{XGXM2} mutants, where brk^{F124} is a known *brk* null mutant carrying an amino acid substitution in the Brk DNA binding domain that abolishes its activity (Fig. 12C,D), confirming $brk^{KO\text{-}w+}$ as a true *brk* KO.

2.2.1.2 Molecular validation of $brk^{KO\text{-}w+}$

The $brk^{KO\text{-}w+}$ candidates were verified by restriction digest and sequencing genomic DNA amplified by PCR, using primers unique to the donor construct and those outside of the genomic regions used in the targeting construct to ensure that the *brk* locus engineered is indistinguishable from the wild-type other than presence of the desired modifications (Fig. 13A).

2.2.1.3 Generation of $brk^{KO\text{-}w-}$ (brk^{KO})

Cre expression was induced in a verified $brk^{KO\text{-}w+}$ line to get rid of the *white* marker and generate the $brk^{KO\text{-}w-}$ (brk^{KO}) where the endogenous *brk* gene was replaced by ØC31 bacteriophage *attP* and a *loxP* site. The brk^{KO} was then re-verified genetically and molecularly (Fig. 13B) as described above [see sub sub-sections 2.2.1.1 and 2.2.1.2].

This was followed by the integration of a wild-type *brk* gene at the *attP* site in brk^{KO} to generate brk^{rescue} as described below in sub-section 2.2.2. The molecular and functional rescue of the KO locus in the brk^{rescue} was assessed as described below [see sub-subsection 2.2.1.4]. Brk protein levels were also evaluated in the brk^{KO} and brk^{rescue} homozygous clones in the wing disc

as indicated below [see sub-subsection 2.2.1.5].

2.2.1.4 Validation of *brk^{KO}* by its rescue with the wild-type *brk* gene

Consistent with *brk* being essential for viability *brk^{KO}* strains are embryonic lethal. Integration of wild-type *brk* gene into the *attP* site of *brk^{KO}* restores viability and the resultant *brk^{rescue}* line was validated molecularly (Fig. 13C,D). To test whether *brk^{rescue}* is functionally equivalent to a wild-type allele it was crossed to a series of known *brk* mutants, *brk^{M68}*, *brk^{F124}*, *brk^{E427}*, a *brk* deficiency (embryonic lethal mutants) and *brk^{XA}* (larval/pupal lethal mutant) kept over the FM7 balancer (which has a wild-type *brk* allele) and the relative survival of the heterozygous *brk^{rescue}/brk^{mutant}* was compared to *brk^{mutant}/FM7* adults that emerged from the same cross; by this analysis *brk^{rescue}* was confirmed to be functionally indistinguishable from the wild-type (Fig. 14).

2.2.1.5 Evaluation of protein levels in *brk^{KO}* and *brk^{rescue}*

Brk protein levels were assessed *in brk^{KO}* and *brk^{rescue}* homozygous clones generated by Flp mediated mitotic recombination in the wing disc (Golic and Lindquist, 1989). Flp mediated mitotic recombination involves the use of Flp Recognition Target (FRT) sites located at identical positions on homologous chromosomes. Following Flp-FRT site-specific recombination between homologous chromosomes after DNA replication, the daughter chromatids will segregate and the region of the chromosome arm distal to the FRT site will be made homozygous (Fig. 15). To this end *brk^{KO}* and *brk^{rescue}* were recombined onto a chromosome carrying FRT sites close to the centromere and following mitotic recombination homozygous *brk* mutant clones were obtained along with a sister clone of the wild-type allele, the latter being known as a ‘twin-spot’ (Fig. 15). The wild-type chromosome carries an eGFP tag, as a result the twin-spot is marked by double dose of eGFP while the homozygous mutant clone shows complete absence of it (Fig. 15).

Wing discs carrying brk^{KO} and brk^{rescue} clones were stained with an antibody specific for Brk and imaged with care ensuring that the confocal detector was not saturated. Using ImageJ software the average fluorescence within a clone situated in the lateral region was measured along with the average within the adjacent wild-type twin-spot and the relative difference was calculated, a relative value of 1 will then indicate no difference and this was repeated for twenty independently generated mutant clone-twin spot pairs for the mutant and wild-type. In brk^{KO} mutant clones no protein was present (Fig. 16A; note, no perdurance of endogenous Brk in clones), while the Brk levels relative to wild-type in brk^{rescue} clones were not significantly different from 1 (Fig. 16A,B and D).

2.2.2 Generation of brk^{rescue} and $brk^{mutants}$

DNA constructs carrying a wild-type *brk* gene or one of a series of mutants in which the 3R, CiM and GiM elements were mutated or deleted individually or in combination were cloned into integration vectors and were flanked 5' by ØC31 bacteriophage *attB* site and 3' by *loxP* site and a *white* marker. This was integrated into brk^{KO} using ØC31 integrase, the resultant transformants, $brk^{mutant\text{-}w+}$ and $brk^{rescue\text{-}w+}$ were identified by the presence of the *white* marker (red eyes) and validated molecularly to ensure presence of the desired modifications (Fig. 10B and 13C). The *white* marker, *attL* site and extra vector sequences were excised using Cre recombinase resulting in strains carrying either wild-type or mutant *brk* genes that apart from the desired mutations, differ from the native locus only by possessing an *attR* site (50 bp) and a *loxP* (34 bp) in the 5' UTR and 3'UTR, respectively. The final engineered brk^{mutant} lines were verified as described below.

2.2.3 Validation of *brk*^{mutant} strains

2.2.3.1 Molecular validation of *brk*^{mutants}

The *brk*^{mutants} were verified by restriction digest and sequencing genomic DNA amplified by PCR, using primers unique to the donor construct and those outside of the genomic regions used in the construct to ensure that the *brk* locus engineered is indistinguishable from wild-type other than presence of the desired modifications (Fig. 13C,D).

2.2.3.2 Evaluation of protein levels in *brk*^{mutants}

Brk protein levels were assessed *in brk*^{mutant} homozygous clones generated by Flp mediated mitotic recombination in the wing disc as described above [see sub-subsection 2.2.1.5]. The Brk levels relative to wild-type in all *brk* mutants, including *brk*^{3M} in which all three repression domains/motifs are eliminated were found to be not significantly different from 1 (Fig. 16C,D).

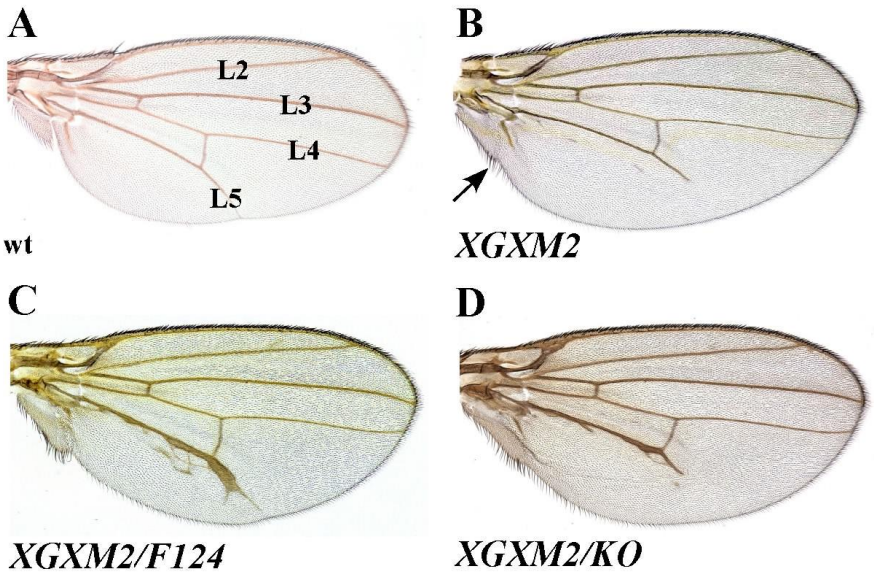

Figure 12: Genetic validation of *brk*^{KO}

(A) Wild-type wing with longitudinal veins indicated, L2-L5. (B) The *brk*^{XGXM2} hypomorph has a slightly enlarged posterior, incomplete L5 and a fused alula (arrowed). (C) The *brk*^{XGXM2} phenotypes are more severe over a null allele, *brk*^{F124}. (D) The *brk*^{XGXM2}/*brk*^{KO} is comparable in severity to the null (*brk*^{F124}) over *brk*^{XGXM2}.

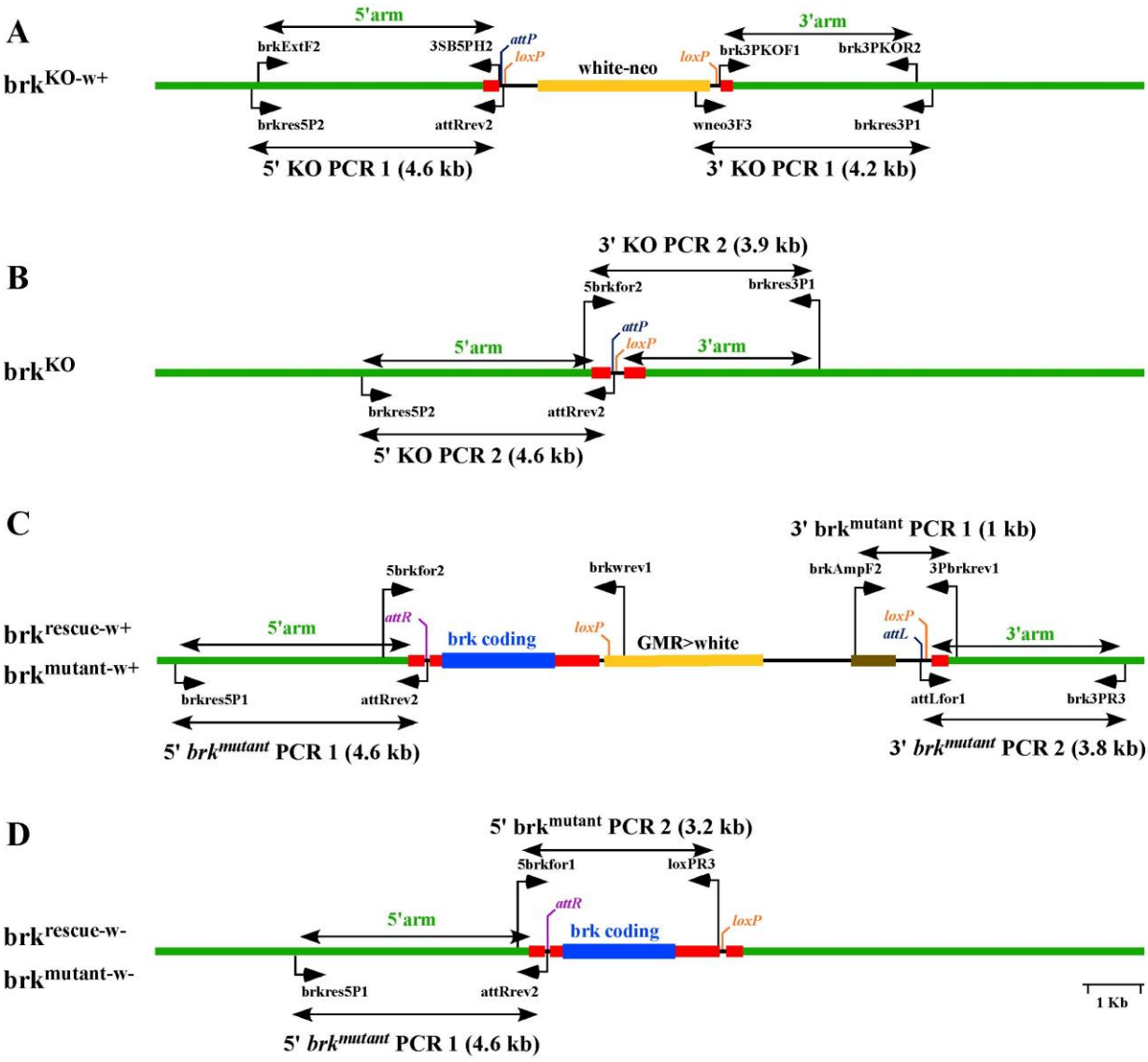

Figure 13: Molecular validation of *brk*^{KO} and endogenous *brk* mutants.

All genotypes indicated were confirmed by PCR amplification of genomic DNA including the novel 5' and 3' ends created by the experimental procedures (expected amplicon size indicated), followed by restriction mapping and sequencing. The PCR was performed with primers outside of those used to generate the arms used in the targeting construct. Validation of the final mutant was also confirmed by amplifying the *brk* gene using a primer in the region including the novel *loxP* sequence in the 3' UTR and a 5' primer outside of the transcription unit.

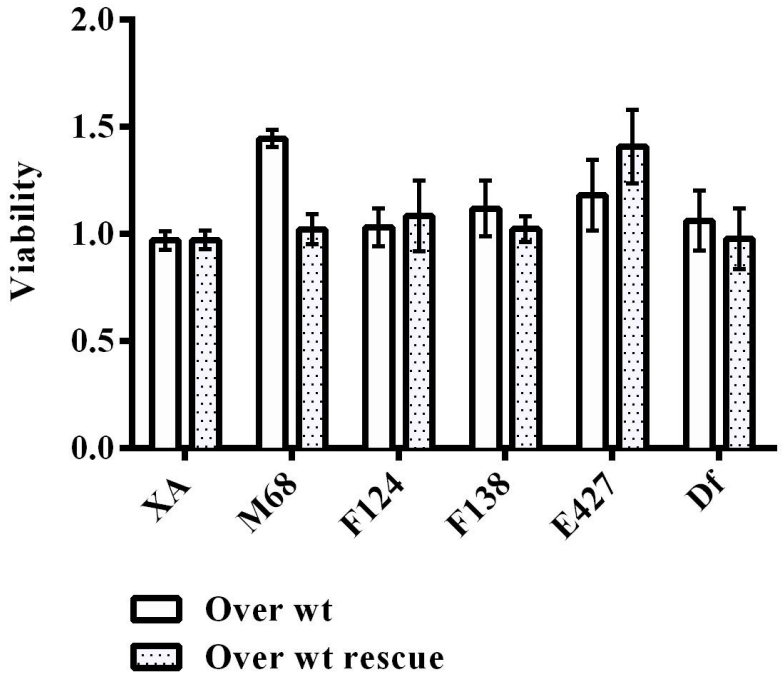

Figure 14: *brk*^{rescue} is functionally equivalent to the wild-type

The viability (number of observed/expected) of *brk*^{rescue} heterozygotes over a series of embryonic lethal *brk* mutants, *brk*^{M68}, *brk*^{F124}, *brk*^{F138}, *brk*^{E427}, a *brk* deficiency (Df) and the larval/pupal lethal *brk*^{XA} was calculated and compared to heterozygotes of a wild-type allele over the same mutants. Although the average is slightly reduced for the *brk*^{rescue} heterozygotes, the difference with the wild-type heterozygotes was not significant (n = 3, in each experiment at least 100 females were evaluated, $P > 0.05$, Mann Whitney U test) indicating that the integration of a wild-type *brk* allele at the deletion locus in the *brk*^{KO} restores the native *brk* locus functionally.

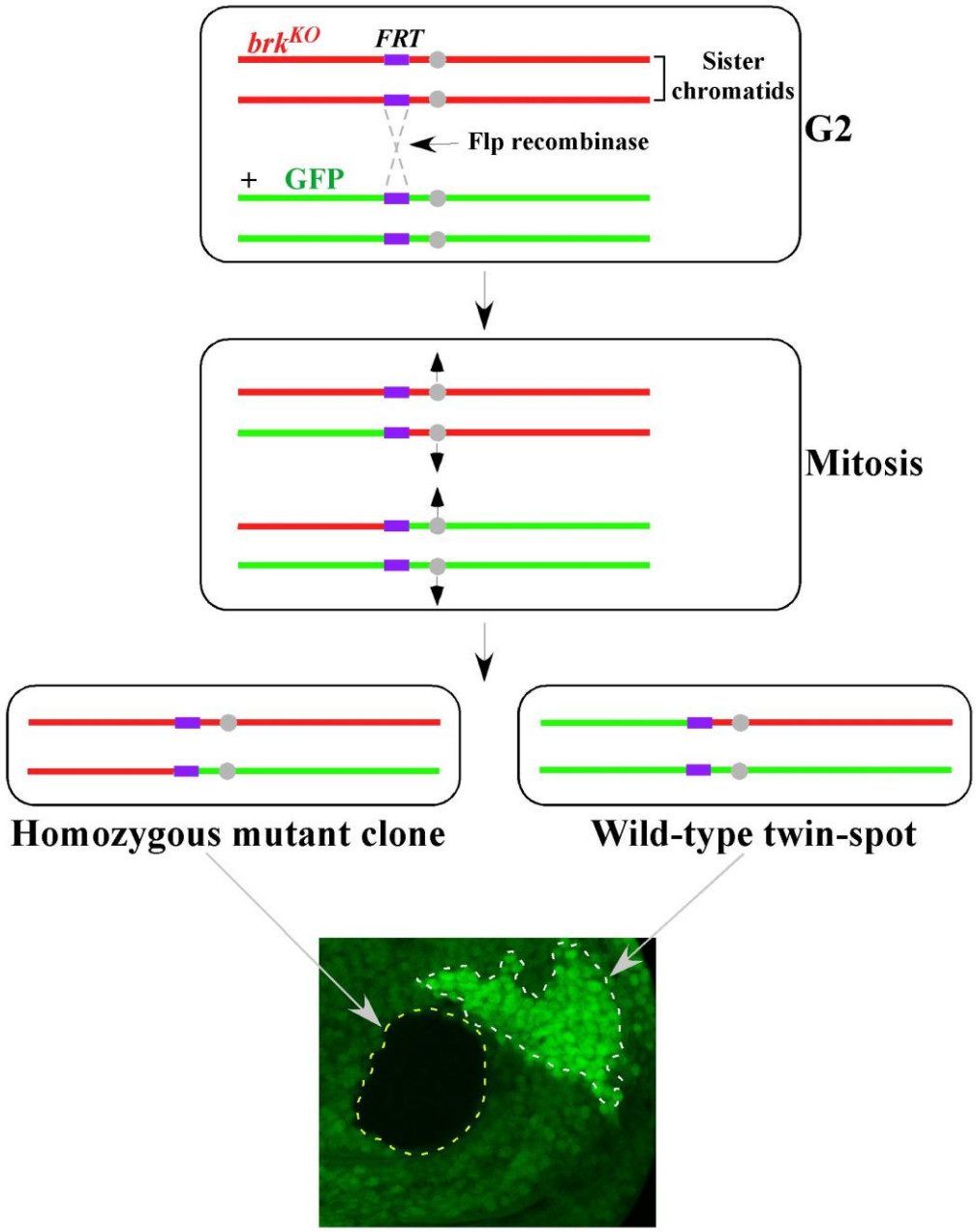

Figure 15: Overview of Flp-FRT mediated mitotic recombination to generate *brk* mutant clones.

The chromosome carrying *brk* mutant allele, for example brk^{KO} is indicated in red and the wild-type chromosome, + was tagged with GFP and is shown in green. Following Flp-FRT site-specific recombination between homologous chromosomes after DNA replication daughter chromatids segregate and the region of the chromosome arm distal to the FRT site is made homozygous. This produces a homozygous mutant clone (yellow dashed line, no GFP expression) and an adjacent sister wild-type twin-spot (white dashed line).

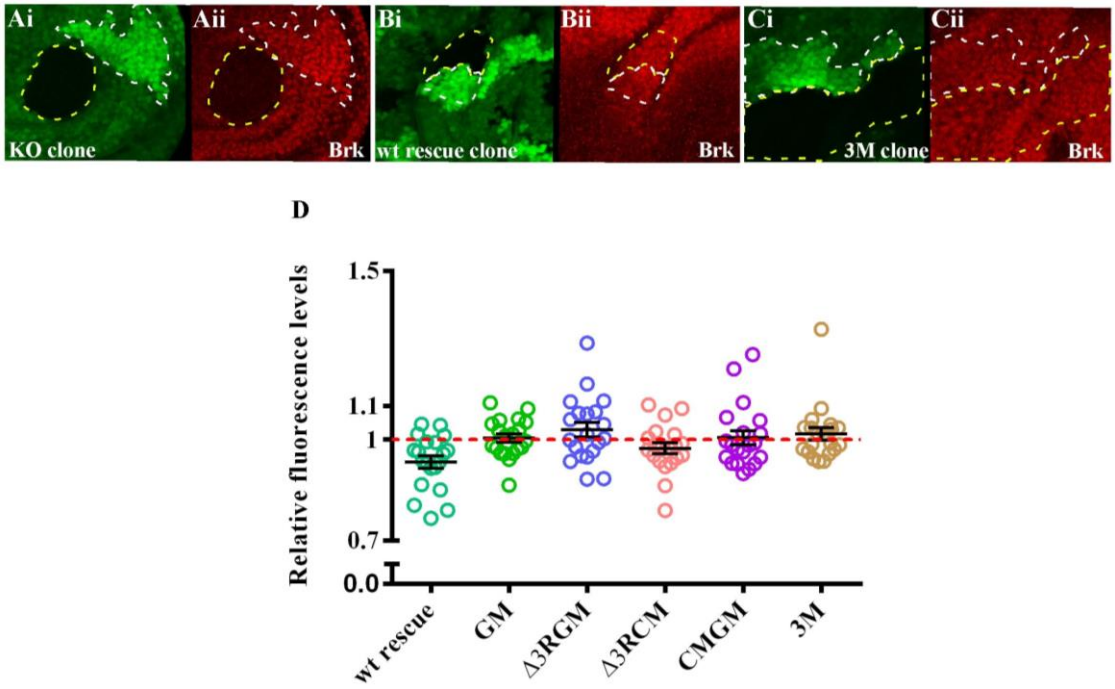

Figure 16: Comparison of Brk protein levels to wild-type in *brk*^{KO}, *brk*^{rescue} and the *brk*^{mutants} generated.

Third instar wing imaginal discs containing homozygous mutant clones marked by loss of GFP and stained for Brk (antibody, red). The levels of Brk in the mutant clone (yellow dashed line) were compared to wild-type Brk protein levels in the adjacent twin spot (white dashed line). (A) *brk*^{KO} clone shows complete absence of Brk. Brk levels in *brk*^{rescue} (B) and *brk*^{3M} (C) clones are indistinguishable from wild-type Brk in adjacent twin-spot. (D) Wing discs carrying clones of the genotypes indicated were stained with Brk antibody, imaged on a confocal microscope taking care that the detector was not saturated. Using ImageJ, the average fluorescence level within a clone situated in the lateral region was measured along with the average within an adjacent wild-type twin spot and the relative difference was calculated; a relative value of 1 will then indicate no difference. As shown in the scatter-plot the twenty mutant Brk flurorescence values relative to wild-type for every *brk* mutant and *brk*^{rescue} do not differ significantly from the expected wild-type value of 1, indicated with red dashed line ($P > 0.05$, chi-square test for trend).

2.2.4 List of *brk* mutants generated

As described above a series of *brk* mutants with CiM, GiM and 3R mutated individually and in combination were generated (Fig. 17).

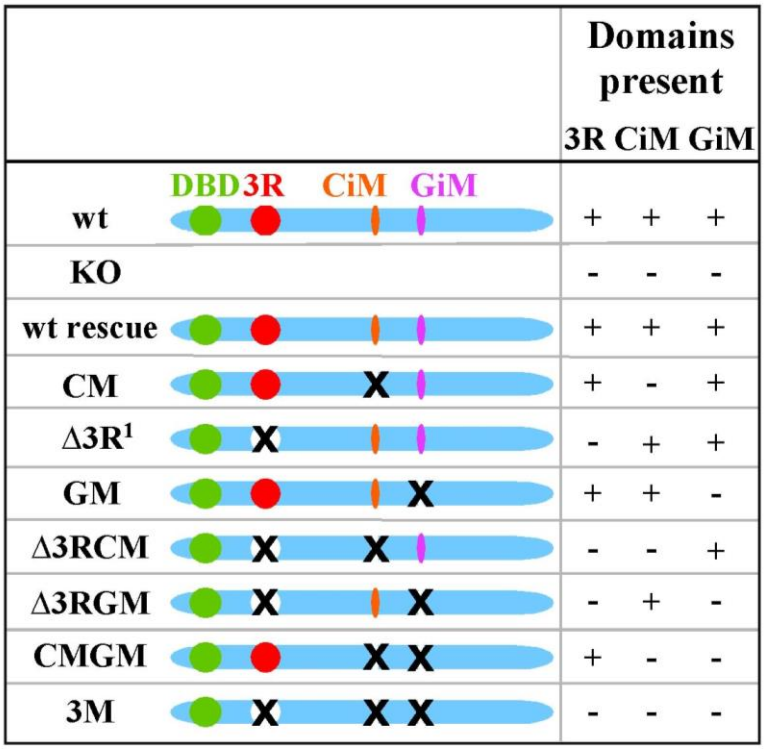

Figure 17: Summary of *brk* mutants generated.

Wild-type (wt) Brk has a DNA binding domain (DBD) and three independent repression/motifs: 3R, CtBP interaction motif (CiM) and Gro interaction motif (GiM).
AW = adult with wild-type morphology; EL = embryonic lethal
[1] Previously, (Winter and Campbell, 2004) this deletion was referred to as NA.

2.2.5 Genetic Validation of *brk* mutants

The *brk* mutant alleles generated were further verified genetically. The brk^{GM} and brk^{CM} mutants are predicted to be unable to recruit Gro and CtBP, respectively, based on previous *in vitro* binding studies along with some genetic support (Hasson et al., 2001; Zhang, H. et al., 2001); however, we wanted to confirm this genetically with our new alleles. If Brk requires a co-repressor to repress a specific target we would expect this target to be derepressed in both a *brk* mutant unable to recruit this co-repressor as well as in a mutant for the co-repressor. To test this rationale a *sal* reporter, salE1, a minimal region driving GFP expression similar to endogenous *sal,* identified in our lab was used as a target in the wing disc (Fig. 18A) for these genetic validation studies because previous studies indicated Brk required either CtBP or Gro to repress this reporter (Winter, 2004). The salE1 enhancer is a 471 bp subfragment of the enhancer element located 10 kb upstream of *salm,* (the *sal* locus consists of two partly redundant genes, *salm* and *salr* (Kuhnlein et al., 1997; Barrio and de Celis, 2004).

The salE1 reporter drives expression medially, in the wing-pouch, albeit in a slightly wider region when compared to the endogenous *sal* (Fig.18A,B), but is repressed laterally by Brk activity as it is derepessed in *brk* null (brk^{M68}) clones in the lateral regions of the wing-pouch (Fig. 18C) (Winter, 2004). However, salE1 does not behave identically to the endogenous *sal* gene, and unlike *sal* its expression is not dependent on Omb (del Alamo Rodriguez et al., 2004) (Campbell, unpublished). Previous analysis of *CtBP* and *gro* single and *CtBP*, *gro* double mutant clones revealed that salE1-GFP expression is derepressed similarly only when both are removed, indicating that either is sufficient to provide Brk with repressive activity to silence salE1, although there does appear to be some minor derepression of expression in *gro* single mutant clones but only very close to the normal expression domain (Fig. 18D-F) (Winter, 2004).

This predicts that salE1-GFP should be repressed in lateral regions even in brk^{CM} and brk^{GM} mutant cells, but that it will be derepressed in the brk^{CMGM} double mutant or in the single mutants if Gro or CtBP, respectively, are also downregulated. In agreement with this prediction in brk^{CM} and brk^{GM} mutant cells salE1-GFP expression appears normal (Fig. 18G,K). But in brk^{CM} mutants when *gro* is knocked down via RNAi in the posterior compartment of the wing disc, salE1-GFP is strongly derepressed (Fig. 18H). Further the salE1 expression also appears wild-type in $brk^{\Delta 3R}$ mutants and remains unaffected when *gro* is downregulated by RNAi in the posterior compartment of the $brk^{\Delta 3R}$ mutant wing disc (Fig. 18I,J). In brk^{CMGM} mutant clones salE1 appears strongly derepressed (Fig. 18L).

These studies are consistent with Brk^{CM} and Brk^{GM} proteins being unable to recruit CtBP and Gro respectively, but indicate that they can recruit the other, while the Brk^{CMGM} protein cannot recruit either co-repressor.

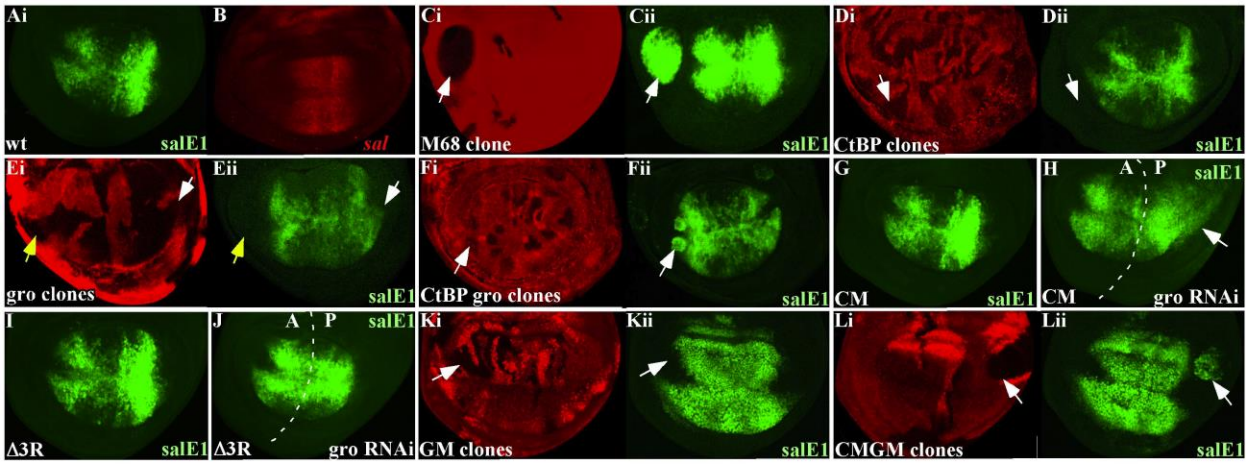

Figure 18: Genetic validation of *brk* mutants.

(A) The salE1 reporter drives GFP expression in a slightly wider domain compared to the endogenous *sal* (antibody, red) expression (B) in wild-type discs. (C) brk^{M68} null mutant clone shows strong derepression of salE1 laterally (arrow). (D) salE1 expression is unaffected in *CtBP* mutant clones. (E) *gro* mutant clones show minor derepression of salE1 in the posterior compartment (white arrow) but it is unaffected anteriorly (yellow arrow). (F) salE1 is strongly derepressed in *CtBP gro* double mutant clones (arrow). (G) In brk^{CM} mutant discs salE1 appears wild-type (A). (H,J) The interface between anterior (A) and posterior (P) compartments in the wing disc is marked by a dashed line. (H) Expression of salE1 is strongly derepressed (arrow) when Gro is knocked down via *gro* RNAi in posterior compartment of the wing disc in the brk^{CM} hemizygotes. (I) In $brk^{\Delta 3R}$ mutant disc salE1 appears wild-type (A). (J) salE1 is unaffected when *gro* is knocked down using *gro* RNAi in the posterior compartment of $brk^{\Delta 3R}$ hemizygotes. (K) salE1 expression is unaffected in brk^{GM} mutant clones (K) salE1 is strongly derepressed (arrow) in brk^{CMGM} mutant clones.

3.0 ANALYSIS OF BRINKER MUTANTS

3.1 INTRODUCTION

Brk functions by recruiting both the co-repressors CtBP and Groucho (Gro) via short 4-10 amino acid recruitment motifs CiM and GiM (CtBP- and Gro-interaction motifs) and in addition it possesses an independent repression domain, 3R that contains a poly-histidine rich region and a stretch of poly-alanine (Fig. 6A) (Winter and Campbell, 2004).

Previous studies aimed at characterizing why Brk possesses multiple repression domains/motifs do not provide strong conclusions, Gro appears to be sufficient for Brk activity in the embryo and the wing disc, thus it is unclear why it recruits CtBP and possesses the additional repression domain, 3R. The Brk CiM is evolutionarily conserved (Winter and Campbell, 2004) further suggesting that it must be important, at least in some contexts. The approaches utilized in the previous studies have several limitations discussed above [see section 1.10]. I hypothesized that the possible reasons why Gro is not sufficient for Brk activity maybe quantitative, qualitative, noise reduction and availability [see section 1.7].

In order to address these possibilities and get around the limitations of the previous studies, I chose to compare Brk activity from mutants in which the CiM, GiM and 3R are nonfunctional. As mentioned previously no such mutants exist for Brk, consequently in this study a series of endogenous Brk mutants with the CiM, GiM and 3R mutated individually

and/or in combination were generated using the genomic engineering technique of Huang *et.al* (Huang et al., 2009) [see chapter 2.0]. Mutant Brk activity was analyzed in the tissues where Brk is known to function [see section 1.8] namely, the early embryo where Brk is expressed ventrolaterally and represses dorsally expressed genes *decapentaplegic (dpp), tolloid (tld), zerknullt (zen)* (Jazwinska et al., 1999a), *schnurri (shn)* and *crossveinless-2 (cv2)* that are derepressed in *brk* mutants; in the late embryonic ventral ectoderm where Brk is ubiquitously expressed and patterns the abdominal denticle belts that are consequently reduced in *brk* mutants (Lammel et al., 2000; Saller et al., 2002); the wing disc where *brk* is expressed in a lateral to medial gradient and regulates medially expressed genes *spalt (sal)* and *optomotor-blind (omb)* that are derepressed in a *brk* mutant (Campbell and Tomlinson, 1999; Jazwinska et al., 1999b; Minami et al., 1999; Muller et al., 2003) and in oogenesis, where Brk is expressed in the follicle cells surrounding the oocyte in a posterior to anterior gradient and patterns egg-shell specializations including dorsal appendages and operculum that are lost and expanded respectively in *brk* mutants (Chen and Schupbach, 2006; Shravage et al., 2007).

A summary of the mutants generated in this study and their activity is shown in Fig. 19.

	Domains present			Viability	Target repression			Denticle Belts[4]	Female Fertility
	3R	CiM	GiM		Wing *sal*	Wing *ombZ*	Embryo[3]		
 wt	+	+	+	AW	+++	+++	+++	wt	wt
KO	-	-	-	EL	-	-	-	xxxx	-
wt rescue	+	+	+	AW	+++	+++	+++	wt	wt
CM	+	-	+	AW	+++	+++	+++	wt	75%
Δ3R[1]	-	+	+	AW	+++	+++	+++	wt	75%
GM	+	+	-	EL	++	++	-	xx	-
Δ3RCM	-	-	+	AW[2]	+++	++	+++	xx	-
Δ3RGM	-	+	-	EL	++	++	-	xxx	-
CMGM	+	-	-	EL	+	+	-	xxx	-
3M	-	-	-	EL	-	-	-	xxxx	-

Figure 19: Summary of activity of *brk* mutants.

Wild-type (wt) Brk has a DNA binding domain (DBD) and three independent repression/motifs: 3R, CtBP interaction motif (CiM) and Gro interaction motif (GiM).
AW = adult with wild-type morphology; EL = embryonic lethal
[1] Previously, (Winter and Campbell, 2004) this deletion was referred to as NA.
[2] Few females survive to adult and many males may have slight defects in wing patterning.
[3] *dpp, tld, zen, shn, cv2*
[4] Severity of loss of ventral embryonic denticles in 1st instar larvae

3.2 RESULTS

3.2.1 Viability and adult phenotype: Gro recruitment is necessary, and CtBP, 3R are not required, to generate a wild-type fly

Previous studies suggested that Gro is the primary co-repressor utilized by Brk and this is confirmed by the mutant analysis in the present study, initially by a simple assessment of the viability of the different alleles. Like null alleles, any of the alleles in which the GiM is mutated, including the brk^{GM} mutant in which the CiM and 3R domain remain intact, are embryonic lethal indicating that Gro recruitment is indispensible for Brk activity to ensure viability of the fly, while CtBP recruitment alone or in combination with the 3R domain are not essential in this respect (Fig. 19).

The brk^{CM} and $brk^{\Delta 3R}$ adults appear morphologically wild-type with a wild-type wing phenotype (Fig. 20A-C), while even some $brk^{\Delta 3RCM}$ mutants, which have Gro as their sole repressive activity (with the exception of a weak repression domain between the CiM and GiM) (Winter and Campbell, 2004) can survive to adulthood with a wild-type phenotype. However, $brk^{\Delta 3RCM}$ is clearly not wild-type, displaying a high degree of lethality, in particular among females that appear to die mostly at the end of embryogenesis or early in larval development; many $brk^{\Delta 3RCM}$ males do actually survive to adult, but at lower rates than wild-type. While most of the $brk^{\Delta 3RCM}$ male survivors had wings with a small but significant posterior enlargement, this phenotype was variable and occasionally the $brk^{\Delta 3RCM}$ wing-width approached and appeared quite close to that of the wild-type (Fig. 20D,E and Fig. 21A,B). Further sometimes but not always $brk^{\Delta 3RCM}$ males had at least one wing that in addition to being posteriorly enlarged had a fused and defective alula (Fig. 20E). Note the area of $brk^{\Delta 3RCM}$ wings did not vary significantly

from that of the wild-type suggesting that Gro activity alone suffices to make an almost morphologically wild-type fly barring small differences (Fig. 21C).

For lethal alleles, i.e. those that are lacking a functional GiM, the requirement of different factors in patterning the adult wing was investigated by analyzing the phenotype of mutant clones at this stage. Previous studies showed that null mutant clones located in the proximal anterior or posterior resulted in outgrowths (Campbell and Tomlinson, 1999) and this is also true for brk^{KO}, brk^{3M}, $brk^{\Delta 3RGM}$ and brk^{CMGM}; however, although brk^{GM} clones are associated with some minor effects on vein patterning they never result in significant outgrowths (Fig. 22B-F).

Taken together this shows that while in some individuals Gro is sufficient for Brk to take a fly from fertilization to what morphologically appears to be a wild-type adult, in others it is not entirely sufficient, thus, CtBP recruitment and 3R are required to ensure that this happens consistently. Additionally in the absence of Gro, CtBP and 3R together can provide significantly more repressive activity than they can individually.

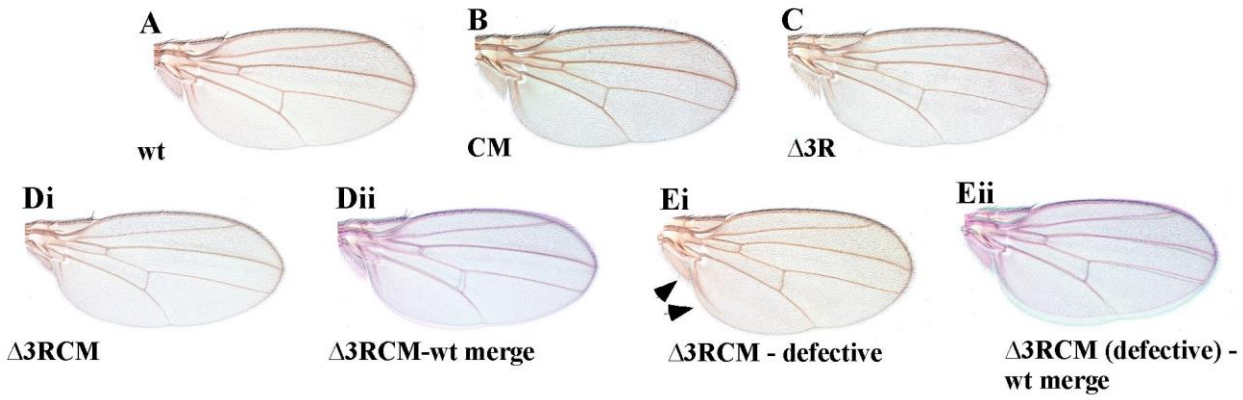

Figure 20: Adult wings from viable *brk* mutants

(A) Wild-type. (B) brk^{CM} and (C) $brk^{\Delta 3R}$ wings appear morphologically wild-type. (D-E) $brk^{\Delta 3RCM}$ hemizygotes often occasionally have morphologically wild-type wings (D) while others are enlarged posteriorly with fused alulae (E).

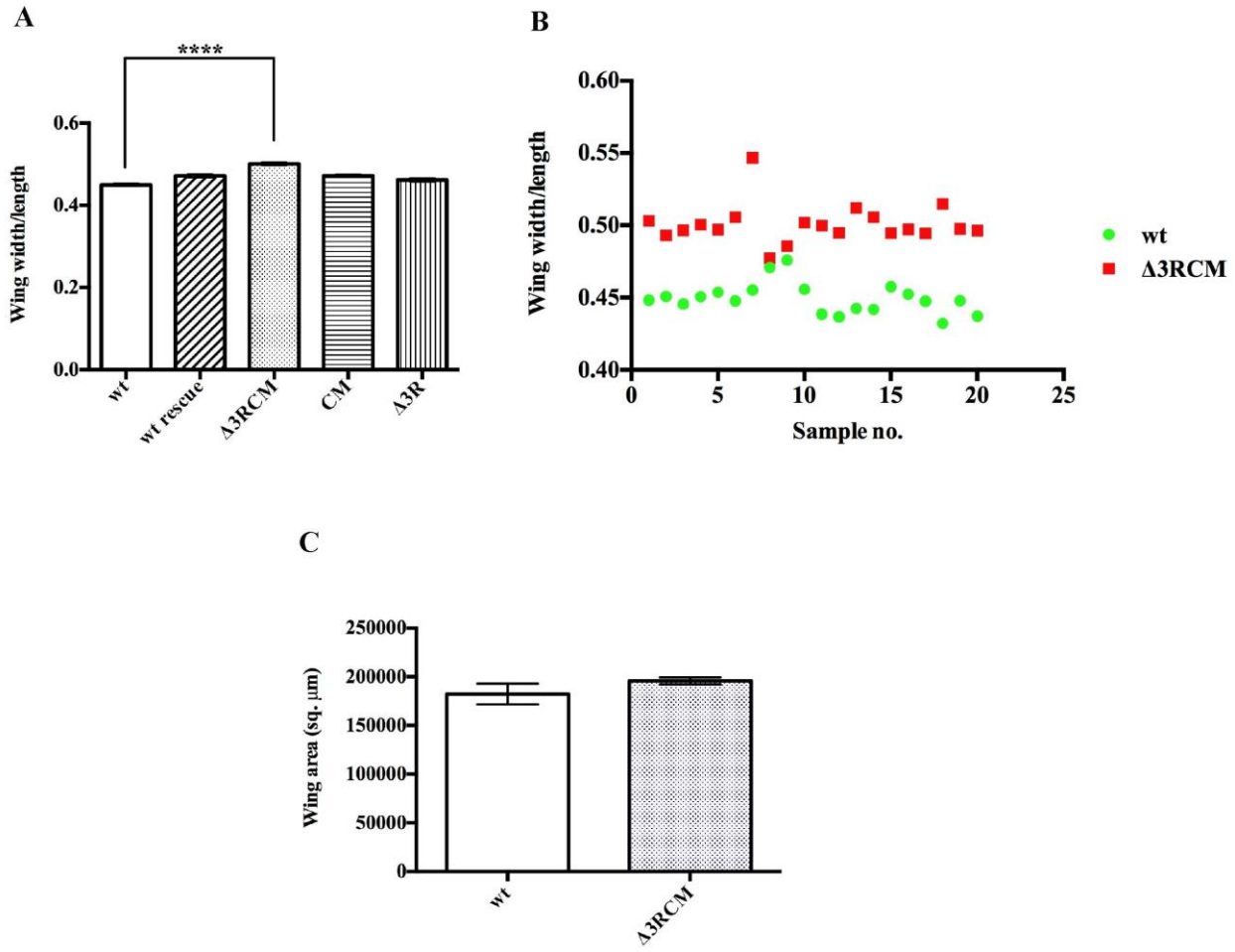

Figure 21: Comparison of adult wing size in the wild type and viable *brk* mutants.

(A,B) Wing width was normalized to its length. Histogram showing that the wing width/length ratio of $brk^{\Delta 3RCM}$ wings is slightly but significantly higher (n = 20, P < 0.0001, Mann Whitney U test) while that of brk^{rescue}, brk^{CM} and $brk^{\Delta 3R}$ is not significantly different when compared to the wild-type (n = 10 for each mutant, P > 0.05, Kruskal Wallis test followed by Dunn's multiple comparison). (B) Scatter-plot of the width/length ratio for $brk^{\Delta 3RCM}$ and wild-type wings. For some $brk^{\Delta 3RCM}$ wings the width/length ratio approaches close to the wild-type while for most others they are higher than the wild-type. (C) The area of $brk^{\Delta 3RCM}$ wings is not significantly different from that of the wild-type (n = 20, P > 0.05, Mann Whitney U test).

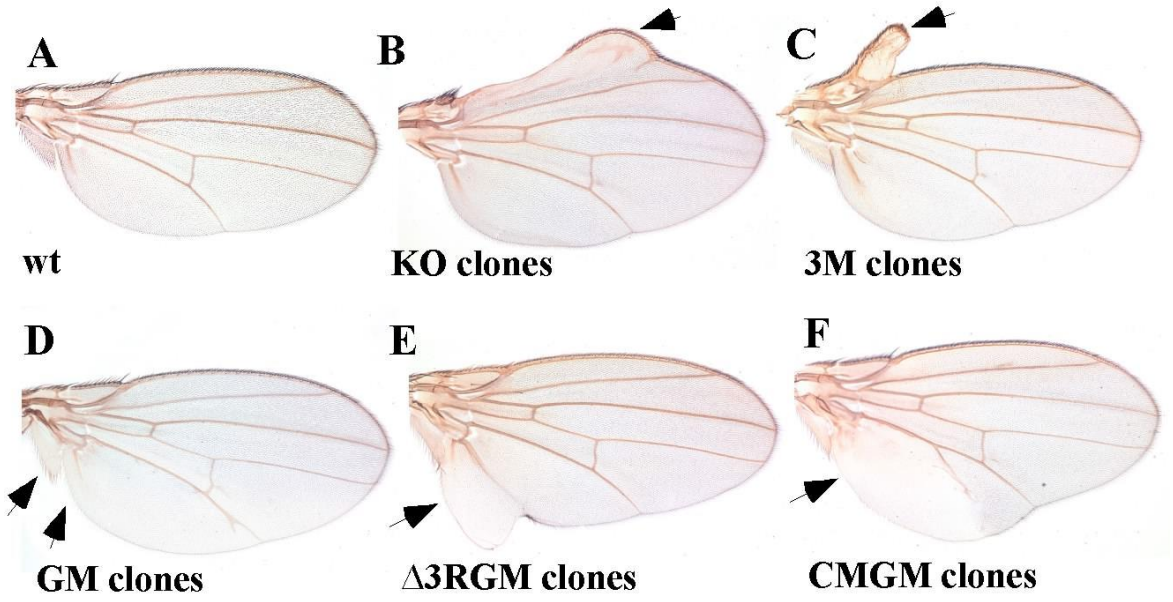

Figure 22: Wing phenotype of embryonic lethal *brk* mutants

Wings from heterozygotes carrying homozygous clones of the brk^{KO} (F), brk^{3M} (G), brk^{GM} (H), $brk^{\Delta 3RGM}$ (I) and brk^{CMGM} (J) alleles positioned in the anterior and posterior proximal region are associated with variable outgrowths (arrowed) apart from brk^{GM} where presence of clones in the alula and along the posterior proximal margin of the wing blade does not produce any obvious defects.

3.2.2 Gro recruitment is required while CtBP and 3R provide limited activity for the repression of wing targets *sal* and *omb*

Using clonal analysis, the ability of mutant Brk proteins to repress the targets *sal* and *omb* in the wing disc was assessed. The endogenous expression of *sal* and *omb* was evaluated using a specific antibody and a lacZ enhancer trap, ombZ respectively; it should be noted that the latter does not appear to completely faithfully reproduce expression of the endogenous gene, but is clearly negatively regulated by Brk (Sivasankaran et al., 2000; Shen et al., 2010). As mentioned earlier, *sal* expression is dependent upon Omb, so it will not behave identically to the reporter

salE1 [see sub-section 2.2.5]. In brk^{KO} and brk^{3M} mutant clones both *sal* and ombZ are strongly derepressed with ectopic expression of the latter inside and outside the wing pouch while ectopic *sal* is restricted to the wing pouch, as demonstrated previously for *brk* null clones (Fig. 23A,B) (Campbell and Tomlinson, 1999; Jazwinska et al., 1999b). Consequently, the $brk^{\Delta 3RCM}$, $brk^{\Delta 3RGM}$, and brk^{CMGM} mutants will reveal the sufficiency of a single factor, namely Gro, CtBP and 3R respectively to provide Brk with repressive activity to repress *sal* and ombZ.

As already mentioned, $brk^{\Delta 3RCM}$ mutants can survive to adult and in $brk^{\Delta 3RCM}$ wing discs *sal* and ombZ expression appears very similar to that of wild-type (Fig. 23L), although it is difficult to detect subtle differences, which can be revealed in clones. In $brk^{\Delta 3RCM}$ clones, as expected *sal* shows no derepression (Fig. 23E); however ombZ is also often normal in clones it can show some derepression but only very close to the endogenous domain. This indicates that Gro alone provides sufficient activity to fully repress *sal* but does not have quite enough activity to consistently repress ombZ (Fig. 23E). In contrast, CtBP alone provides some activity but is far from sufficient, as there is some *sal* and ombZ derepression in $brk^{\Delta 3RGM}$ clones but only within the wing pouch close to their endogenous domains (Fig. 23F); so $Brk^{\Delta 3RGM}$ has sufficient activity to repress both *sal* and ombZ in lateral regions but not more medially (Fig. 23F). 3R is even less efficient with brk^{CMGM} clones showing more extensive derepression of both *sal* and ombZ within the wing pouch and ombZ occasionally, but not always, outside of the pouch (Fig. 23G,H). The derepression of ombZ is a little surprising as this was not observed in *CtBP*, *gro* double mutant clones or the brk^{F138} mutant, which encodes a truncated protein eliminating the CiM and GiM (Winter and Campbell, 2004). The reason for this is unclear. This double mutant analysis reveals that CtBP and 3R can provide some activity but are not sufficient for complete repression of wing targets while Gro is sufficient for *sal* but not quite for ombZ.

The *sal* and ombZ expression is normal in discs from brk^{CM} and $brk^{\Delta 3R}$ mutants (Fig. 23I-K), as would be expected as they survive to adults with wild-type wings, indicating neither CtBP nor 3R is required for repression of these targets. However, Gro is necessary as both are derepressed in brk^{GM} clones, but only close to the endogenous domains (Fig. 23C,D). In this respect, brk^{GM} is less severe than either brk^{CMGM} or $brk^{\Delta 3RGM}$, indicating CtBP and 3R together provide Brk with more activity than either alone in the absence of Gro.

Taken together this data indicated that although Gro is required for both *sal* and ombZ regulation and is completely sufficient for *sal* but not quite for ombZ. Together CtBP and 3R appear to provide increased repressive activity in absence of Gro. Also contrary to previous findings that indicated that 3R alone is sufficient to repress ombZ (Hasson et al., 2001; Winter and Campbell, 2004) the current study revealed that at physiological levels it is sufficient occasionally, only in the lateral regions of the wing disc where levels of Brk are high.

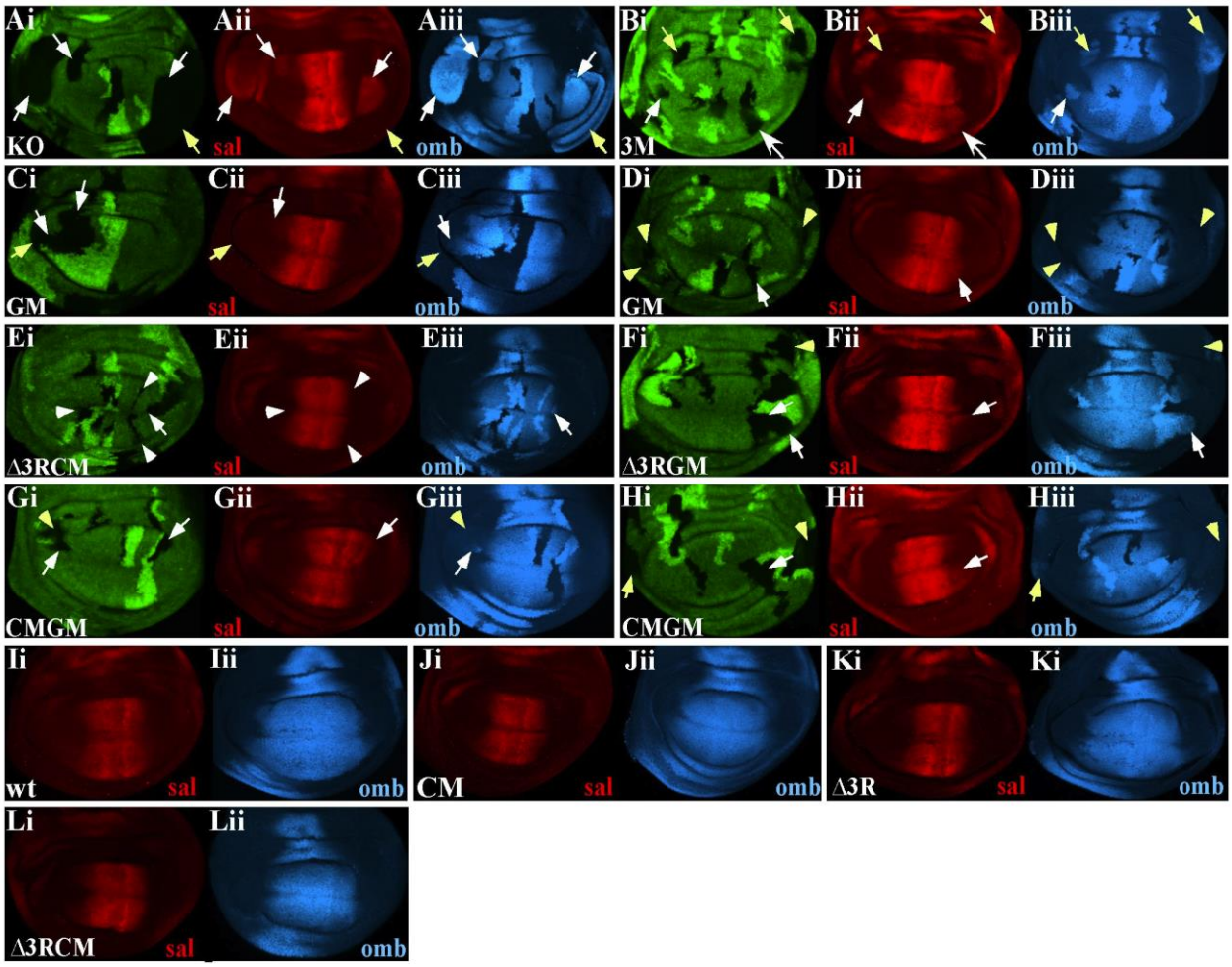

Figure 23: *sal* and *omb* expression in *brk* mutants.

(A-H) Third instar wing discs containing mutant clones, marked by the loss of a ubiquitous GFP transgene, and stained for *omb-lacZ* (βGal antibody; note *omb* is on the same chromosome as *brk* so its expression is lost in twin-spots) and Sal (antibody), anterior (left). (A,B) In brk^{KO} and brk^{3M} clones *sal* and *omb-lacZ* are both strongly deregulated with ectopic expression of *omb-lacZ* outside the wing-pouch/hinge (yellow arrows) while ectopic *sal* is restricted to the wing-pouch/hinge (white arrows). (C,D) In brk^{GM} mutant clones *sal* and *omb-lacZ* are derepressed close to their endogenous domain (white arrows) but not more laterally within (yellow arrows) or outside the wing-pouch/hinge (yellow arrowheads). (E) In $brk^{\Delta 3RCM}$ clones *sal* is not derepressed (white arrowhead) but minor expansion of *omb-lacZ* is noted (white arrow). (F) In $brk^{\Delta 3RGM}$ mutant clones located mediolaterally both *sal* and *omb-lacZ* are derepressed (white arrows) but no ectopic expression is seen outside the wing-pouch/hinge (yellow arrowhead). (G,H) In brk^{CMGM} mutant clones *sal* is derepressed within the wing-pouch alone (white arrows) and *omb-lacZ* is derepressed close to its endogenous domain (white arrow) but not more laterally (yellow arrowhead). (Hi,iii) Sometimes *omb-lacZ* is derepressed outside the wing-pouch/hinge (yellow arrow) but not always (yellow arrowhead). (I) Expression of *sal* and *omb-lacZ* in wild-type wing-discs. In brk^{CM} (J), $brk^{\Delta 3R}$ (K) and $brk^{\Delta 3RCM}$ (L) mutant discs *sal* and *omb-lacZ* appear wild-type (I).

3.2.3 Gro recruitment is required and sufficient for Brk to negatively regulate itself

Brk has been shown previously to negatively regulate its own expression in the region of the wing disc where its expression is graded and this appeared to be dependent on either CtBP or Gro (Hasson et al., 2001; Moser and Campbell, 2005). As with brk^{F124} null mutant clones (Moser, 2008), Brk is upregulated in brk^{3M} clones located mediolaterally in the wing disc where Brk expression is graded (Fig. 24A). To analyze the sufficiency of Gro, CtBP and 3R individually for Brk auto-regulation, clones with the double mutants were generated. Brk is upregulated in brk^{CMGM} and $brk^{\Delta 3RGM}$ clones located mediolaterally indicating that neither CtBP nor 3R are sufficient for Brk to repress itself (Fig. 24C,D). In contrast, it is not derepressed in similarly positioned $brk^{\Delta 3RCM}$ mutant clones in the wing-disc (Fig. 24E) suggesting that Gro is sufficient for Brk to negatively regulate itself.

To test the necessity of Gro, CtBP or 3R, Brk expression in the single mutant clones was assessed. As expected Brk expression appears wild-type in brk^{CM} and $brk^{\Delta 3R}$ mutant wing discs (Fig.25A-C) confirming neither CtBP nor 3R are necessary if Gro is recruited. Brk is sometimes but not always deregulated in brk^{GM} mutant clones positioned mediolaterally in the wing-disc where Brk expression is graded (Fig. 24B), indicating that Gro recruitment is necessary sometimes but not always sufficient for Brk mediated negative auto-regulation. Further together CtBP and 3R can provide Brk with almost sufficient activity to repress itself but not quite.

The insufficiency of CtBP and 3R alone in this regard is also demonstrated by knocking down Gro in in brk^{CM} and $brk^{\Delta 3R}$ mutant wing discs, a situation essentially the same as found in brk^{CMGM} and $brk^{\Delta 3RGM}$ clones. As already mentioned, Brk expression is normal in brk^{CM} and $brk^{\Delta 3R}$ mutant discs, but when Gro is knocked down via RNAi in the posterior compartment of brk^{CM} and $brk^{\Delta 3R}$ mutant discs, Brk is strongly derepressed (Fig. 25D,E).

In conclusion, it appears that Gro is sufficient for Brk to repress itself, and while individually CtBP and 3R are insufficient, together they are almost capable of providing Brk with sufficient activity. Previous, suggestions that CtBP was sufficient were based on analysis of *gro* mutant clones in which Brk was not derepressed (Hasson et al., 2001); however, as shown here, this is because Brk can utilize both CtBP and 3R under these circumstances, not only CtBP.

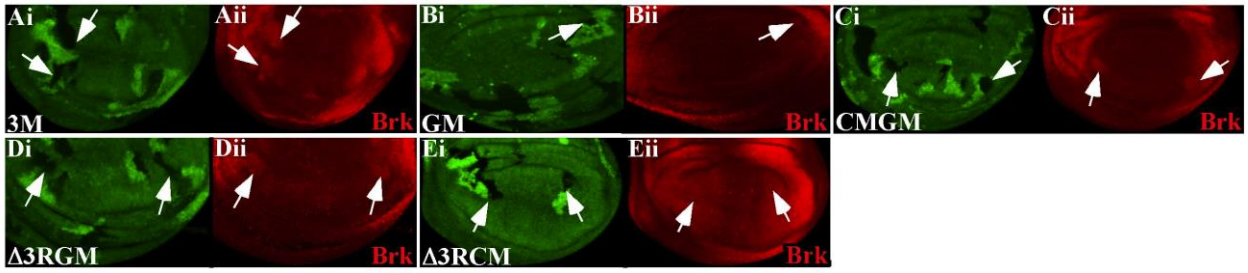

Figure 24: Gro is required and is mostly sufficient for Brk to negatively regulate itself

Third instar wing discs containing mutant clones, marked by the loss of a ubiquitous GFP transgene and stained for Brk (antibody, red). Brk expression was evaluated in mutant clones situated mediolaterally in the wing-disc where its expression is graded and was compared to wild-type Brk in adjacent twin spots. (A) brk^{3M} mutant clones show strong upregulation of Brk when compared to the wild-type (arrow). (B) Some brk^{GM} mutant clones show a minor derepression of Brk (arrow). Both brk^{CMGM} (C) and $brk^{\Delta 3RGM}$ (D) mutant clones show strong upregulation of Brk (arrows). (E) $brk^{\Delta 3RCM}$ mutant clones do not show any deregulation of Brk.

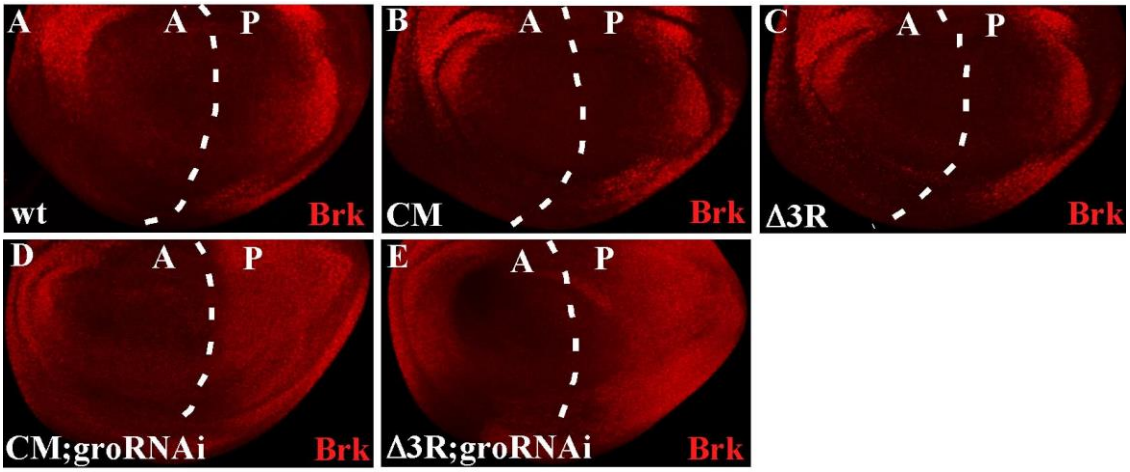

Figure 25: CtBP or 3R alone are not sufficient for Brk to negatively regulate itself.

Third instar wing discs stained for Brk (antibody, red). The interface between anterior (A) and posterior (P) wing disc compartments is demarcated by white dashed line. (A) Brk expression in a wild-type wing disc. (B) In brk^{CM} and (C) in $brk^{\Delta 3R}$ mutant wing discs Brk expression appears wild-type. When Gro is knocked down via RNAi in the posterior compartment of a brk^{CM} (D) and $brk^{\Delta 3R}$ (E) mutant wing disc Brk is strongly derepressed confirming that individually neither 3R nor CiM can enable Brk mediated negative regulation of its own expression.

3.2.4 Gro is necessary and sufficient for regulation of the early embryonic Brk targets

Early in embryonic development Brk is required to restrict the expression of several genes along the dorsoventral axis (Jazwinska et al., 1999a). It is expressed ventrolaterally in late syncytial and early cellular blastoderm stage embryos (Fig. 26A) and dorsally expressed genes such as *dpp*, *tld*, *zen*, *shn* and *cv2* show expansion in *brk* mutants (Jazwinska et al., 1999a) (H.Ashe, unpublished). In brk^{KO} and brk^{GM} embryos all Brk targets are derepressed and their expression expands ventrally (Fig. 26B-D). In contrast, in early $brk^{\Delta 3RCM}$ embryos expression of these targets appears wild-type (Fig. 26E). Thus, Gro is required and appears to be sufficient for Brk activity in early embryogenesis.

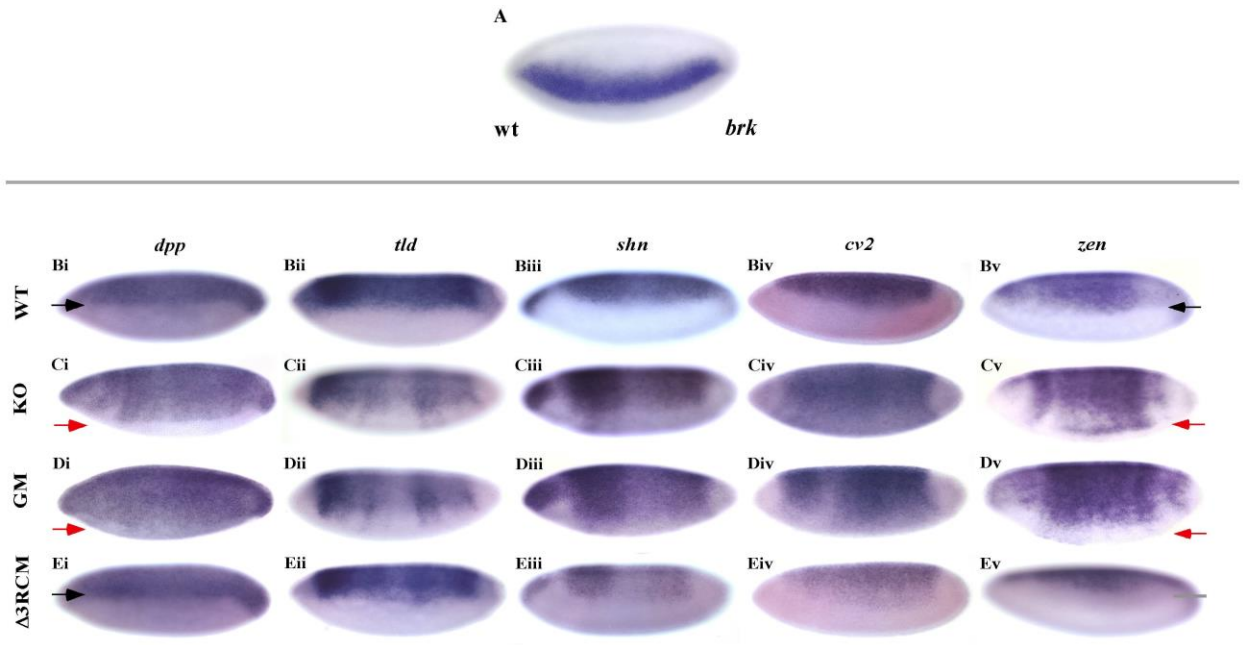

Figure 26: Gro is required and sufficient to repress D/V patterning genes.

Cellular blastoderm embryos hybridized with probes against *brk*, *dpp*, *tld*, *shn*, *cv2* and *zen;* anterior (left), dorsal (up). (A) In wild-type embryos *brk* is expressed ventrolaterally. (B) In wild-type embryos the targets are expressed only dorsally (black arrows). (C) In brk^{KO} embryos targets are derepressed strongly (red arrows). (D) In brk^{GM} embryos the targets are derepressed and ventrally expanded as in brk^{KO} embryos (C). (E) In $brk^{\Delta 3RCM}$ embryos the targets do not show ventral expansion.

3.2.5 Gro is necessary but not quite sufficient for Brk activity in late embryogenesis

Brk is also required later in embryogenesis in the abdominal epidermis where it helps to establish the repeating pattern of cuticular denticle belts (VDBs). Each denticle belt is formed in the anterior region of each segment and is composed of six rows of denticles, that are hair-like protrusions of the cuticle which also exhibit a characteristic polarity with those in rows 1 and 4 pointing anteriorly while the rest point posteriorly (Fig. 27A) (Saller et al., 2002). The VDBs in *brk* null mutants are severely reduced as there is a significant loss of denticles and all remaining denticles exhibit a polarity defect and point posteriorly (Fig. 27B,F,G) (Jazwinska et al., 1999a; Lammel et al., 2000; Saller et al., 2002). Both the brk^{KO} and brk^{3M} (Fig. 27C,F,G) have a very similar phenotype to that previously described for null mutants. In contrast, the brk^{GM} cuticle displays a less severe phenotype, so that although all remaining denticles point posteriorly and the VDB width is significantly narrower compared to wild-type, they are wider than in the brk^{KO} or brk^{3M} mutants, suggesting that while Gro is required for Brk activity in the ventral embryonic ectoderm it is not completely sufficient and CtBP and the 3R domain must provide some activity (Fig. 27D,F,G). Consistent with this observation, $brk^{\Delta 3RCM}$ mutants also display a mild cuticle phenotype with some loss of denticles, from the first three rows, but polarity of the remaining denticles is unchanged (Fig. 27E-G). Thus, Gro appears to be required and largely sufficient for Brk activity in patterning the VDBs and that CtBP and 3R are needed together to facilitate its full repressive activity.

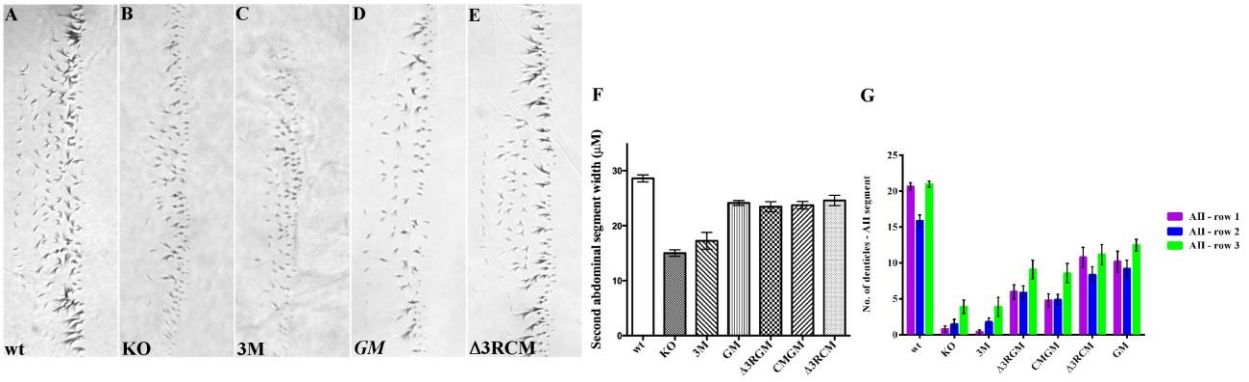

Figure 27: Second abdominal denticle belt phenotype in *brk* mutants

(A) The wild-type belt consists of six rows of denticles, those in rows 1 and 4 point anteriorly while the rest point posteriorly. (B and C) In brk^{KO} and brk^{3M} mutants the denticle belts are significantly reduced, with loss of most denticles from the rows 1-3 and all remaining denticles pointing posteriorly. (D) In brk^{GM} mutants this phenotype is less severe although all remaining denticles point posteriorly. (E) The denticle belts in most $brk^{\Delta 3RCM}$ are narrower, although occasionally their width appears close to wild-type accompanied by a minor loss of denticles but in all $brk^{\Delta 3RCM}$ mutants most remaining denticles retain the wild-type polarity. (F) The second abdominal denticle belt (AII) is significantly narrower in all *brk* mutants compared to wild-type ($n = 10$, $P < 0.01$, Mann Whitney U test). (G) Comparison of the number of denticles in rows 1-3 of (AII) in *brk* mutants and the wild-type. Number of denticles remaining in rows 1-3 of AII ventral denticle belt are in significantly reduced in the following mutants, brk^{KO}, brk^{3M}, brk^{CMGM}, $brk^{\Delta 3RGM}$, brk^{GM} and $brk^{\Delta 3RCM}$ ($n = 10$, $P < 0.01$, Mann Whitney U test). The loss of denticles is most severe in brk^{KO} and brk^{3M}, of intermediate severity in and brk^{CMGM} and $brk^{\Delta 3RGM}$ mild in brk^{GM} and $brk^{\Delta 3RCM}$ mutants. All mutants except $brk^{\Delta 3RCM}$ display a polarity defect, such that all the remaining denticles point posteriorly compared to the wild-type where denticles in rows 1 and 4 point anteriorly while rest point posteriorly.

3.2.6 Wingless and Rho maybe directly or indirectly regulated by Brinker in the late embryonic ventral ectoderm

Brk is expressed ubiquitously in the late ventral embryonic ectoderm (Fig. 28A) but how it patterns the VDBs in the abdominal epidermis is not clear. Denticle formation is promoted by EGFR signaling and antagonized by Wingless (Wg) signaling, the latter promoting smooth cuticle formation (Bejsovec and Martinez Arias, 1991; Szuts et al., 1997; Sanson, 2001). Wg and the transmembrane protease Rhomboid (Rho), which promotes EGFR signaling by processing the EGFR ligand Spitz, are both expressed in single stripes within each segment (Golembo et al., 1996; Alexandre et al., 1999; Lee et al., 2001). The stripes of *wg* were expanded and the stripes *of rho* expression were reduced respectively in brk^{KO} embryos (Fig. 28B-E), suggesting that *wg* could be a direct Brk target and an as yet unidentified Brk target, in the late ventral ectoderm, could regulate *rho*. Brk is ubiquitously expressed in the ventral ectoderm (Fig. 28A) so how it spatially restricts the expression of these two genes remains to be determined.

Taken together the loss of Rho and expansion of Wg expression in brk^{KO} ventral embryonic ectoderm is consistent with and explains the loss of denticles in *brk* mutants.

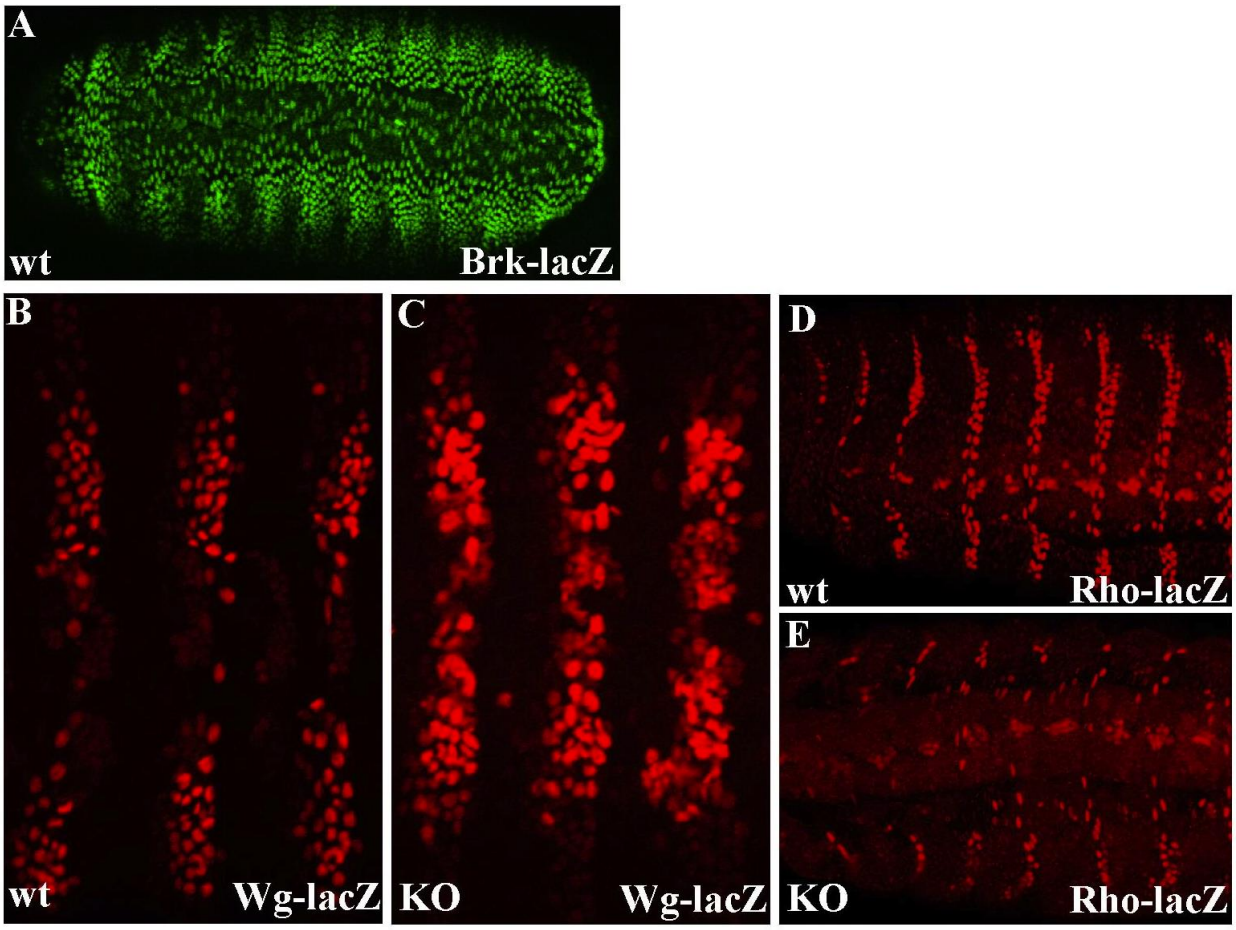

Figure 28: Wingless and Rhomboid expression in *brk*^{KO} late ventral ectoderm.

(A) Brk (lacZ) is expressed ubiquitiously in the wild-type ventral embryonic ectoderm (stage 12-13). (B,C) Wingless (Wg, lacZ) expression in the late ventral embryonic cuticle, stage 12-13. (B) Wild-type Wg expression. In *brk*^{KO} mutants (C) the Wg stripes are expanded in comparison to wild-type (B). (D,E) Rhomboid (Rho, lacZ) expression in the late ventral embryonic ectoderm, stage 12-13. (D) Wild-type Rho expression. (E) In *brk*^{KO} mutants Rho expression was significantly reduced when compared to the wild-type (D).

3.2.7 Relative viability of *brk* mutants that survive to adult

brk^{CM} and $brk^{\Delta 3R}$ mutants are fully viable in the lab, in the sense that a healthy homozygous stock can be established and easily maintained. Some $brk^{\Delta 3RCM}$ mutants can survive to adult but few females survive and a viable stock cannot be established. To determine how Brk activity in these three mutants compares I evaluated the viability of the wild-type and these mutants (brk^{test}) over a series of known embryonic lethal *brk* mutants namely brk^{KO}, brk^{M68} and brk^{F138} that are embryonic lethal mutants and brk^{XA}, a larval/pupal lethal mutant that survives embryogenesis. The viability ($brk^{test}/brk^{known\ mutant}$) was compared to the number of brk^{test}/FM7 (FM7 balancer carries wild-type *brk* gene) obtained in the same cross. The viability of brk^{CM} and $brk^{\Delta 3R}$ mutants was significantly reduced over embryonic lethal *brk* mutants but was comparable to the wild-type over brk^{XA} (Fig. 29). In contrast $brk^{\Delta 3RCM}$ survivors displayed significantly reduced viability over all known mutants tested (Fig. 29). This is consistent with the results of the relative viability of these mutants over known *brk* mutants, with $brk^{\Delta 3RCM}$ appearing worse than brk^{CM} and $brk^{\Delta 3R}$ in its ability to rescue brk^{XA} mutant that survives through embryogenesis.

Thus, together this data indicates that even though these mutants survive to adult, Brk activity is obviously compromised, most significantly over the double mutant.

3.2.8 Male fertility in viable *brk* mutants

Assessment of male fertility revealed that 69% of $brk^{\Delta 3RCM}$ males tested were infertile, while the percentage of infertile brk^{CM} and $brk^{\Delta 3R}$ males are indistinguishable from wild-type (Fig. 30). Further analysis is required to understand the cause of the infertility observed in $brk^{\Delta 3RCM}$ males. Currently it is not known if Brk activity is required for spermatogenesis but the increased male

infertility in $brk^{\Delta 3RCM}$ warrants further scrutiny to understand if this deficiency is correlated to $Brk^{\Delta 3RCM}$ mutant activity. If so this further substantiates that while some $brk^{\Delta 3RCM}$ male survivors appear almost morphologically wild-type they are not entirely so functionally and that Gro alone, while largely sufficient to pattern the adult fly, may be deficient in some respects.

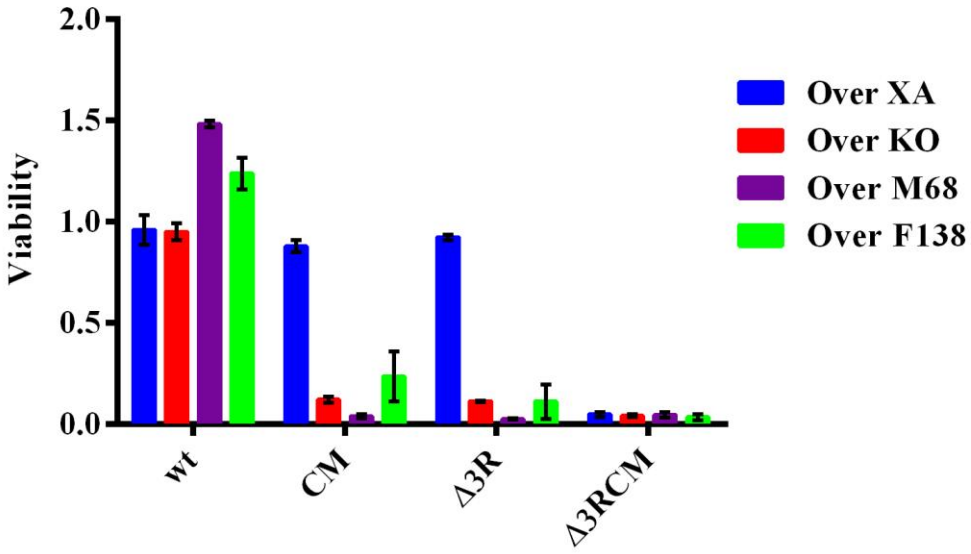

Figure 29: Brk activity in viable mutants brk^{CM}, $brk^{\Delta 3R}$ and $brk^{\Delta 3RCM}$ is compromised

The viability of *brk* mutants, brk^{CM}, $brk^{\Delta 3R}$ and $brk^{\Delta 3RCM}$ over larval/pupal lethal mutant, brk^{XA} and a series of embryonic lethal mutants, brk^{KO}, brk^{M68} and brk^{F138} was assessed by calculating the number of brk^{test} /$brk^{known\ mutant}$ heterozygotes obtained compared to the number of females that were brk^{test} over a wild-type *brk* allele. In each experiment a 100 females were counted. Compared to the wild-type the viability of brk^{CM} and $brk^{\Delta 3R}$ over all embryonic lethal *brk* mutants tested was significantly reduced ($n = 2$, $P < 0.05$, Mann Whitney U test) while they are indistinguishable from the wild-type in their viability over brk^{XA}, a larval/pupal lethal mutant ($n = 2$, $P > 0.05$, Mann Whitney U test). The viability of the double mutant $brk^{\Delta 3RCM}$ was significantly reduced over all *brk* mutants evaluated ($n = 2$, $P < 0.05$, Mann Whitney U test).

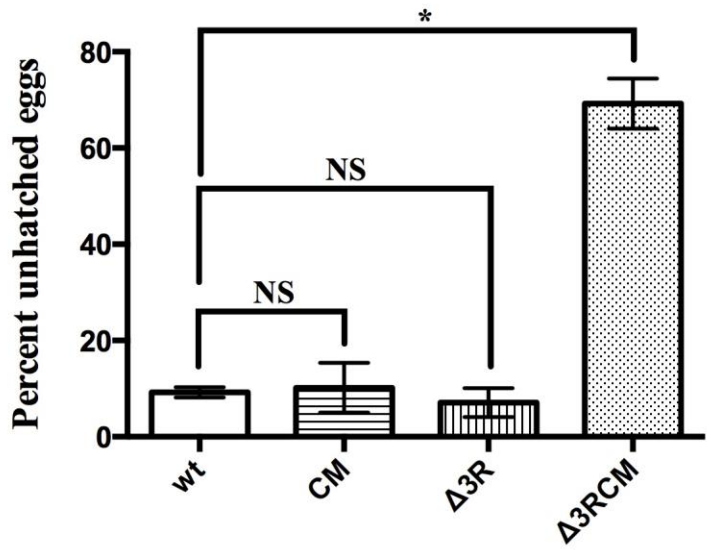

Figure 30: Male fertility is reduced in *brk*$^{\Delta 3RCM}$ mutant

3-4 day old males of each viable *brk* mutant line, *brk*^{CM}, *brk*$^{\Delta 3R}$, *brk*$^{\Delta 3RCM}$ and the wild-type were crossed individually to 3 wild-type females and the number of crosses with unhatched eggs was assessed. A significant number of *brk*$^{\Delta 3RCM}$ males appear infertile (n = 4, in each experiment 50 *brk*$^{\Delta 3RCM}$ males were crossed to wild-type females, $P < 0.05$, Mann Whitney U test). The *brk*^{CM} and *brk*$^{\Delta 3R}$ male fertility does not appear to be significantly different from wild-type (n = 4, in each experiment 50 males of every genotype were crossed to wild-type females, $P > 0.05$, Mann Whitney U test).

3.2.9 Fertility of adult viable *brk* mutants

Further analysis revealed that the adult viable mutants showed defects in oogenesis, first by identifying that they have a reduced fertility with mutant mothers laying a significant number of unfertilized eggs: 29% from *brk*^{CM} mothers and 23% from *brk*$^{\Delta 3R}$ mothers compared to only 5% from wild-type mothers (Fig. 31). As already mentioned, few female *brk*$^{\Delta 3RCM}$ mutants survive to adult. Initially, I was able to attempt a fertility test with three *brk*$^{\Delta 3RCM}$ mutant mothers and found that 38 out of 41 eggs laid remained unfertilized. This suggests that the fertility defect is even

more severe in the double mutant where most of the eggs laid by *brk*$^{\Delta 3RCM}$ females remain unfertilized. However, I have been unable to repeat this assay as homozygous *brk*$^{\Delta 3RCM}$ females are obtained only very occasionally, if at all. However, the heterozygous *brk*$^{\Delta 3RCM}$ mothers (*brk*$^{\Delta 3RCM}$/+) are not significantly different from the wild-type mothers in terms of the number of unfertilized eggs laid by them (Fig. 31) indicating the female fertility defect noted in *brk*$^{\Delta 3RCM}$ homozygous females is rescued by a wild-type cope of *brk* gene.

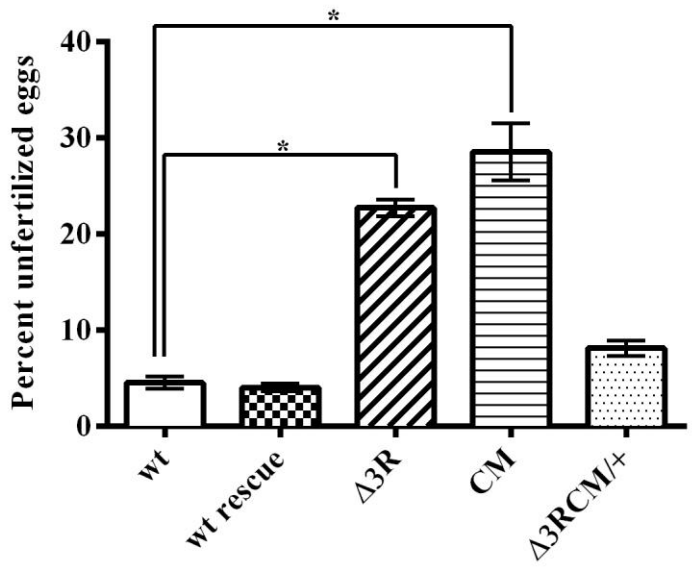

Figure 31: Female fertility in viable mutants *brk*^{CM}, *brk*$^{\Delta 3R}$ and *brk*$^{\Delta 3RCM}$ is compromised

In comparison to wild-type, *brk*^{CM} and *brk*$^{\Delta 3R}$ mothers lay significantly more unfertilized eggs (n = 3, for every experiment at least 100 eggs were evaluated 3-6 hour after egg laying by DAPI staining to determine the number of eggs fertilized for each strain, $P < 0.05$, Mann Whitney U test). While the fertility of *brk*^{rescue} and transheterozygous *brk*$^{\Delta 3R}$/+ mothers is indistinguishable from wild-type (n = 3, for every experiment 100 eggs were evaluated as described above for each, $P > 0.05$, Mann Whitney U test).

3.2.10 CtBP and 3R are required for Brk activity in egg-shell patterning during oogenesis

The reduced fertility observed in brk^{CM}, $brk^{\Delta 3R}$ and $brk^{\Delta 3RCM}$ mothers led me to investigate defects, if any in the morphology of the eggs laid by these mutants to determine if that could explain their reduced fertilization rates. Key features of *Drosophila* eggs are located in the dorsal anterior: the dorsal appendages, a pair of tubes aiding in respiration, the operculum, a lid like structure through which the larva hatches out and the micropyle, an anterior cone shaped structure that allows sperm entry (Berg, 2005).

Brk is required for patterning the operculum and dorsal appendages as the former is enlarged and the latter absent in eggs derived from mothers in which *brk* null clones were generated in the follicle cells responsible for patterning these egg shell structures (Chen and Schupbach, 2006). As expected, the same egg phenotypes were obtained with brk^{KO} mutant clones, but an additional phenotype was also noted: a reduced micropyle, indicating Brk activity is also required for patterning this structure (Fig. 32B, 33). Eggs laid by females carrying brk^{3M} follicle cell clones also appear identical to the brk^{KO} with the complete absence of dorsal appendages, significantly enlarged operculum and a severely stunted micropyle (Fig. 32C, 33).

Eggs laid by brk^{CM} and $brk^{\Delta 3R}$ mothers exhibit similar but milder egg-shell defects when compared to the null including significantly shorter dorsal appendages and a shorter micropyle but possess a wild-type operculum (Fig. 32F,G and 33). Since few $brk^{\Delta 3RCM}$ female survivors reached adulthood, I generated $brk^{\Delta 3RCM}$ clones in the follicular epithelium and analyzed the resultant eggs, which displayed more severe phenotypes, including an enlarged operculum and a significant reduction or occasional absence of dorsal appendages, expanded operculum and a reduced micropyle (Fig. 32D,E and 33). The phenotype of some eggs obtained from females with $brk^{\Delta 3RCM}$ follicle cell clones (Fig. 32E) was more extreme than eggs laid by single mutant

mothers (Fig. 32F,G) but less severe than the brk^{KO} mosaic eggs (Fig. 32B). Occasionally the phenotypes of eggs obtained from females carrying $brk^{\Delta 3RCM}$ follicle cell clones do appear comparable in severity to eggs laid by females carrying brk^{KO} follicle cell clones (Fig. 32B,D). This suggests that 3R and CiM provide most, if not all Brk mediated activity during oogenesis. The reduced micropyle phenotype appears identical in brk^{CM}, $brk^{\Delta 3R}$, $brk^{\Delta 3RCM}$ and brk^{KO} eggs and may account for the reduced fertility if it disrupts sperm access to the egg. Together this data imply that 3R and CtBP facilitate Brk mediated repressive activity and enable correct egg-shell patterning during oogenesis.

3.2.11 Gro is insufficient for Brk activity in oogenesis

Eggs laid by females with brk^{GM} follicle cell clones appear largely wild-type with the operculum mildly different (Fig. 32H and 33), indicating that Gro provides very little activity during oogenesis. However eggs from females with follicle cell clones of the *gro* null allele, gro^{MB36} (Jennings et al., 2008), do possess some patterning defects including reduced dorsal appendages and a reduced micropyle but no significant change in the operculum length (Fig. 33, 34B). Importantly while the gro^{MB36} eggs have shorter dorsal appendages when compared to the wild-type, these are significantly longer when compared to eggs laid by brk^{CM} and $brk^{\Delta 3R}$ mothers (Fig. 34B,D,E), that display a mild egg-shell defect (Fig. 32F,G and 33). However, the fact that the defect in gro^{MB36} eggs is not mirrored by the brk^{GM} eggs suggests that Gro maybe utilized by other transcription factors in egg-shell patterning. Taken together these data indicate that Gro is insufficient for Brk activity in patterning egg-shell membranes and that the 3R domain and CtBP are required in this context.

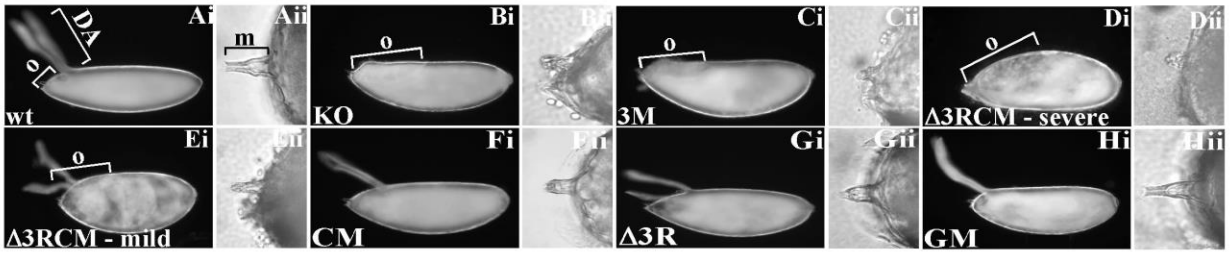

Figure 32: Egg-shell phenotypes of *brk* mutants.

Dorsal appendages (DA), operculum (o) and micropyle (m) are egg-shell structures derived from the follicle cells. (A) Wild-type egg-shell features. (B) Eggs from mothers carrying brk^{KO} mutant clones show loss of DAs, expansion of the operculum and a defective micropyle that is significantly reduced in length. (C) Eggs from mothers carrying brk^{3M} clones show a similar phenotype to brk^{KO} mosaic eggs. (D-E) Eggs from mothers carrying $brk^{\Delta 3RCM}$ clones. (D) $brk^{\Delta 3RCM}$ eggs occasionally appear similar in phenotype to brk^{KO} eggs. (E) Although mostly $brk^{\Delta 3RCM}$ eggs possess an expanded operculum and significantly shorter DAs that appear more severe than eggs laid by brk^{CM} and $brk^{\Delta 3R}$ mothers but less drastic when compared to the brk^{KO} mosaic eggs. Eggs from brk^{CM} (F) and $brk^{\Delta 3R}$ (G) mutants have reduced DAs and micropyle; operculum length remains unchanged from wild-type. (H) Eggs from mothers carrying brk^{GM} mutant clones have a largely wild-type phenotype.

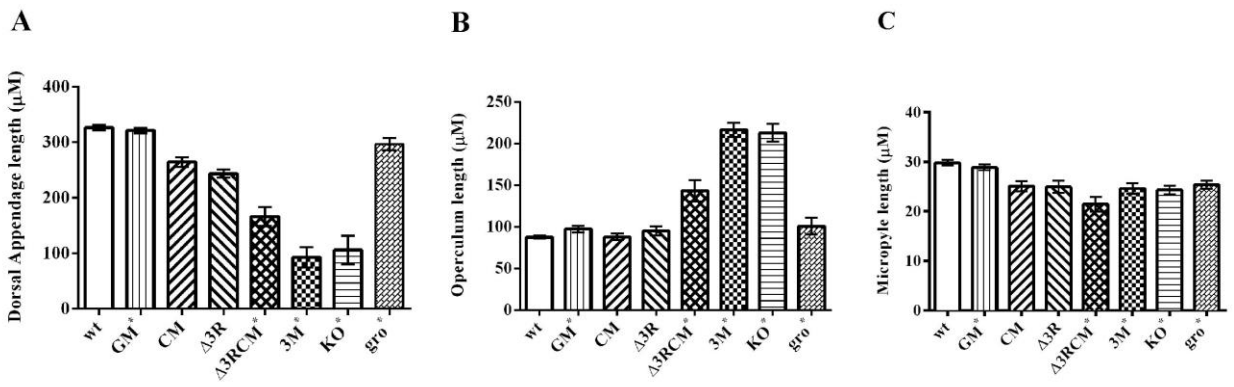

Figure 33: Size of the dorsal appendages (DAs), operculum and micropyle in eggs laid by *brk* mutant mothers or mothers carrying follicle cell clones.

Eggs laid by mothers with follicle cell clones are marked with * Compared to wild-type, eggs from mothers carrying brk^{KO} and brk^{3M} follicle cell clones have (A) only one reduced or no DAs ($P < 0.0001$), (B) the opercula are significantly larger ($P < 0.0001$) and (C) the micropyle are significantly reduced ($P < 0.01$). Eggs from brk^{CM} and $brk^{\Delta 3R}$ homozygous mothers have (A) significantly shorter DAs ($n = 10$, $P < 0.0001$), (B) opercula appear wild-type ($n = 10$, $P > 0.05$) and (C) micropyle are reduced ($n = 10$, $P < 0.01$). Eggs from mothers carrying $brk^{\Delta 3RCM}$ follicle cell clones have (A) significantly shorter or no DAs ($P < 0.0001$) occasionally being as severe as brk^{KO}, (B) opercula that are significantly expanded ($P < 0.0001$) and (C) reduced micropyle ($P < 0.01$). Eggs from mothers carrying brk^{GM} follicle cell clones have (A) wild-type DAs ($P > 0.05$), (B) slightly expanded opercula ($P < 0.05$) and (C) wild-type micropyle ($P > 0.05$). Eggs from mothers carrying follicle cell clones of the *gro* null allele, gro^{MB36} have (A) shorter DAs compared to wild-type ($n = 10$, $P < 0.01$), (B) wild-type opercula ($n = 10$, $P > 0.05$) and (C) reduced micropyle ($n = 10$, $P < 0.01$). P values calculated using the Mann Whitney U test, $n = 20$ unless otherwise indicated.

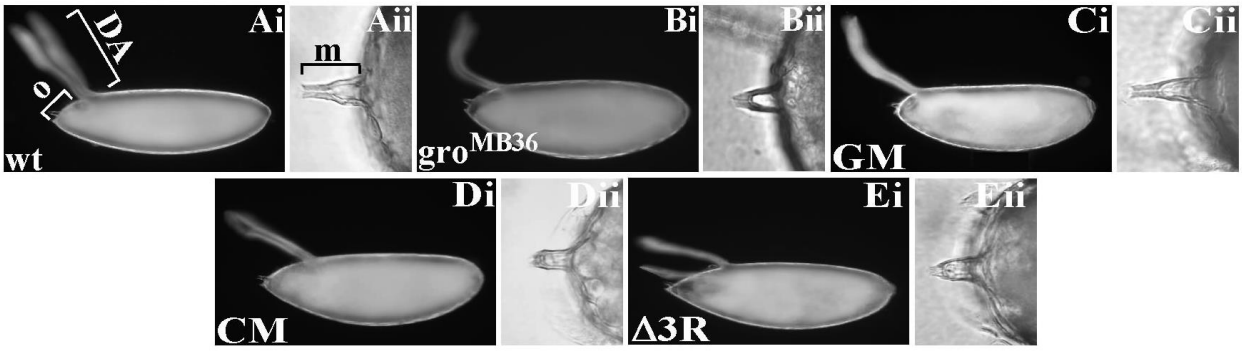

Figure 34: Gro null mutant (*gro*^{MB36}) has a weak defective egg-shell phenotype

Anterior left, dorsal up. Compared to the (A) wild-type (B) the *gro*^{MB36} (*gro* null) mosaic eggs have a weak defective egg-shell phenotype with shorter dorsal appendages (DA) and micropyle (m) but the operculum (o) appears wild-type. The DAs in *gro*^{MB36} eggs are significantly longer than those in the mildly defective eggs laid by (D) *brk*^{CM} and (E) *brk*$^{\Delta 3R}$ mothers. (C) *brk*^{GM} mosaic eggs appear largely indistinguishable from the wild-type.

3.2.12 Gro is phosphorylated and mostly unavailable for Brk activity in oogenesis

Why is Gro not sufficient to provide Brk with activity during oogenesis? Given that Gro activity can be downregulated by MAPK phosphorylation via Receptor Tyrosine Kinase signaling, including EGFR signaling (Hasson et al., 2005; Cinnamon and Paroush, 2008), Brk expression, EGFR signaling activity and Gro phosphorylation were examined during oogenesis. Consistent with previous reports both Brk expression and EGFR signaling were found to be highest in the dorsoanterior follicle cells (Chen and Schupbach, 2006; Shravage et al., 2007) that are patterned by EGFR (Schupbach, 1987) and Dpp signaling (Twombly et al., 1996; Peri and Roth, 2000); dorsoanterior follicle cells include two groups of dorsolateral follicle cells that will each give rise to a dorsal appendage (Dorman et al., 2004; Berg, 2005; Yakoby et al., 2008) and the centripetal cells, the anterior most oocyte-follicle cells resting on the oocyte-nurse cell border that at stage 10 plunge inwards towards the border cells and eventually give rise to the operculum and

micropyle (Edwards and Kiehart, 1996; Yakoby et al., 2008). In tune with previous observations in brk^{KO} mosaic eggs the dorsal appendages are lost, operculum is significantly expanded (Chen and Schupbach, 2006; Shravage et al., 2007), additionally I noted that the micropyle is also defective (Fig. 32B and 33) [see sub-section 3.3.10] confirming that Brk must be critical for the proper patterning of dorsoanterior egg-shell structures.

In wild-type stage 10 egg chambers Brk is expressed in all the follicle cells surrounding the developing oocyte, but at highest levels in the dorsal anterior with levels grading off away from this region (Fig. 35A). EGFR signaling is also high in this region, as monitored by expression of the target, Kek (Fig. 35B). Using an antibody that primarily recognizes the active, unphosphorylated form of Gro, its staining is found to be mirroring that of Kek, with significantly reduced levels in the dorsal anterior consistent with Gro being phosphorylated and its activity levels reduced in the region exhibiting highest levels of EGFR signaling (Fig. 35C).

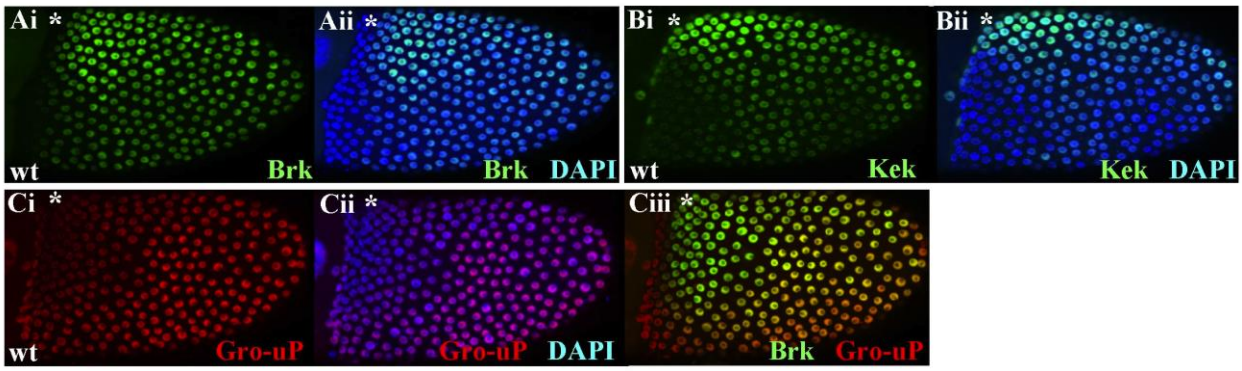

Figure 35: Gro phosphorylation by EGFR signaling in the follicular epithelium.

(A-C) Stage 10 egg chambers, anterior left, dorsal up, * marks the dorsal anterior. (A) In the wild-type, Brk (*lacZ*, ß-Gal) is expressed in a gradient with highest levels in the dorsal anterior follicle cells. (B) Kek (*lacZ*, ßGal), a reporter of EGFR/MAPK signaling is expressed in a similar gradient. (C) An antibody largely specific to the unphosphorylated form of Gro (Gro-uP) shows reduced staining levels in the dorsal anterior marked by highest levels of Brk (Ciii).

3.2.13 Gro is regulated by EGFR signaling in oogenesis

To confirm that EGFR signaling was controlling the patterns of Gro phosphorylation in oogenesis, EGFR signaling was upregulated by driving the expressing of a weakly constitutively active form of MAPK, UAS-rlSEM using GR1-Gal4 and CY2-Gal4 driver lines and in both cases unphosphorylated Gro was ubiquitously reduced (Fig. 36A,B). Upon downregulating EGFR signaling by Ras RNAi using CY2-Gal4 and tub>CD2>Gal4 drivers unphosphorylated Gro was ubiquitously increased (Fig. 36C,D).

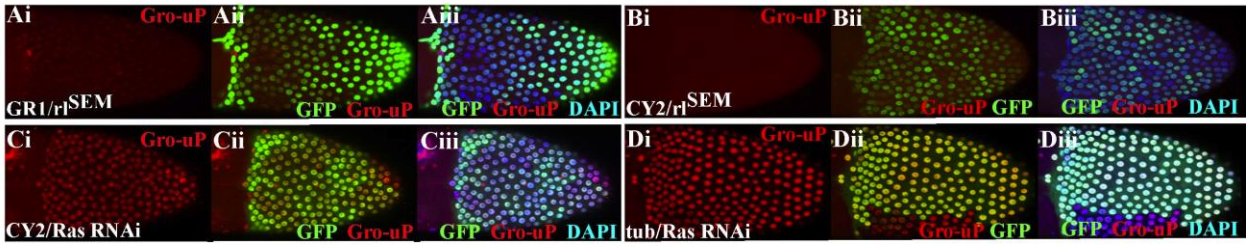

Figure 36: Gro phosphorylation by EGFR signaling in the follicular epithelium.

Stage 10 egg chambers, anterior left, dorsal up. Upregulation of EGFR/MAPK signaling by (A) (GR1/UAS-rl^{SEM}) and (B) (CY2/UAS-rl^{SEM}) leads to ubiquitously reduced unphosphorylated Gro (detected through a largely specific antibody, Gro-uP, red). (Aii,Bii) Follicle cells expressing rl^{SEM} due to GR1-Gal4 (Aii) and CY2-Gal4 (Bii) activity are marked by expression of a UAS-GFP transgene. (Aiii-Diii) Nuclei are marked by DAPI. Merge. Knockdown of EGFR/MAPK signaling by (CY2/UAS Ras85D RNAi) (Ci) and (tubGal4 /UAS Ras85D RNAi) (Di) produces uniform levels of unphosphorylated Gro. Follicle cells showing CY2-Gal4 (Cii) and tub-Gal4 (Dii) activity marked by expression of a UAS-GFP transgene.

3.2.14 Gro phosphorylation by EGFR signaling attenuates it in other tissues

If the interpretation that Gro phosphorylation by EGFR signaling attenuates it is correct then a prediction would be that in other tissues as well if Brk were unable to recruit CtBP, its activity would be compromised following upregulation of EGFR signaling. Above it has been shown that CtBP or Gro are sufficient for Brk to repress the salE1-GFP reporter in lateral regions of the wing disc (Fig. 18) [see subsection 2.2.5]. Upregulation of EGFR signaling in the posterior compartment of wing discs from wild-type flies using UAS-rl^{SEM} does not result in derepression of salE1-GFP and *omb-lacZ* (Fig. 37A,C) consistent with our prediction because CtBP should provide sufficient activity. Further this results in wings with minor defects in wing-vein patterning and number (Fig. 38C). But in *brk*^{CM} mutants upregulation of EGFR/MAPK signaling in the posterior compartment results in the strong derepression of salE1-GFP and *omb-lacZ* (Fig. 37B,D) and is explained by Brk activity being compromised by reduced levels of active unphosphorylated Gro. The resultant wings show a worsening of wing-vein phenotype with increased number and severe mispatterning of wing-veins (Fig. 38D).

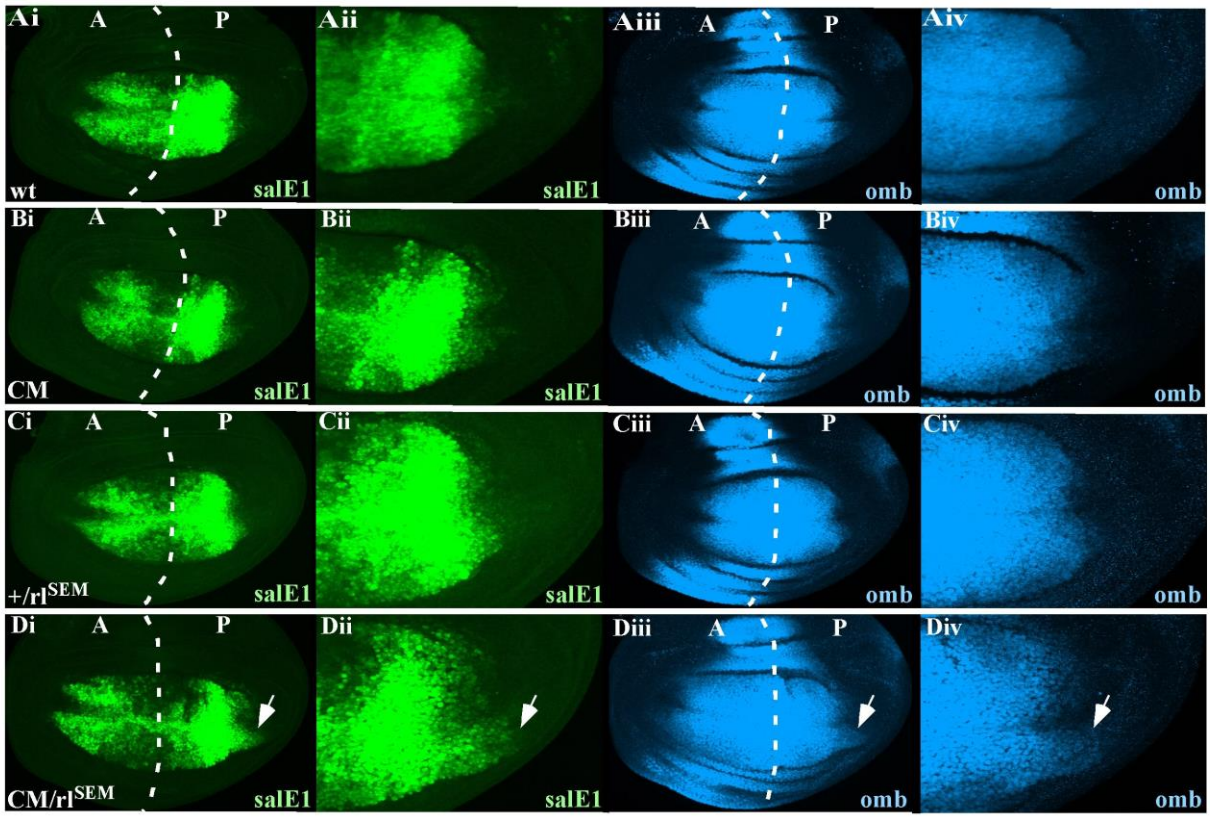

Figure 37: Gro phosphorylation by EGFR signaling attenuates its activity in the third instar wing discs.

Third instar wing discs stained for *omb-lacZ* (βGal antibody, blue) and salE1 expression reported by GFP (green). The boundary between anterior and posterior wing-disc compartments has been indicated by a dashed line. (Ai,ii) Wild-type salE1 (Aiii,iv) Wild-type *omb*. (B) In brk^{CM} mutants the salE1 (Bi,ii) and *omb* (Biii,iv) expression appear wild-type. (C) In wild-type wing-discs the salE1 (Ci,ii) and *omb* (Ciii,iv) expression appears wild-type even after EGFR/MAP signaling upregulation (en-Gal4/UAS-rl^{SEM}) in the posterior compartment of the wing disc. (D) In a brk^{CM} wing-disc both salE1 (Di,ii) and *omb* (Diii,iv) are derepressed following EGFR/MAPK signaling upregulation in the posterior compartment (arrows).

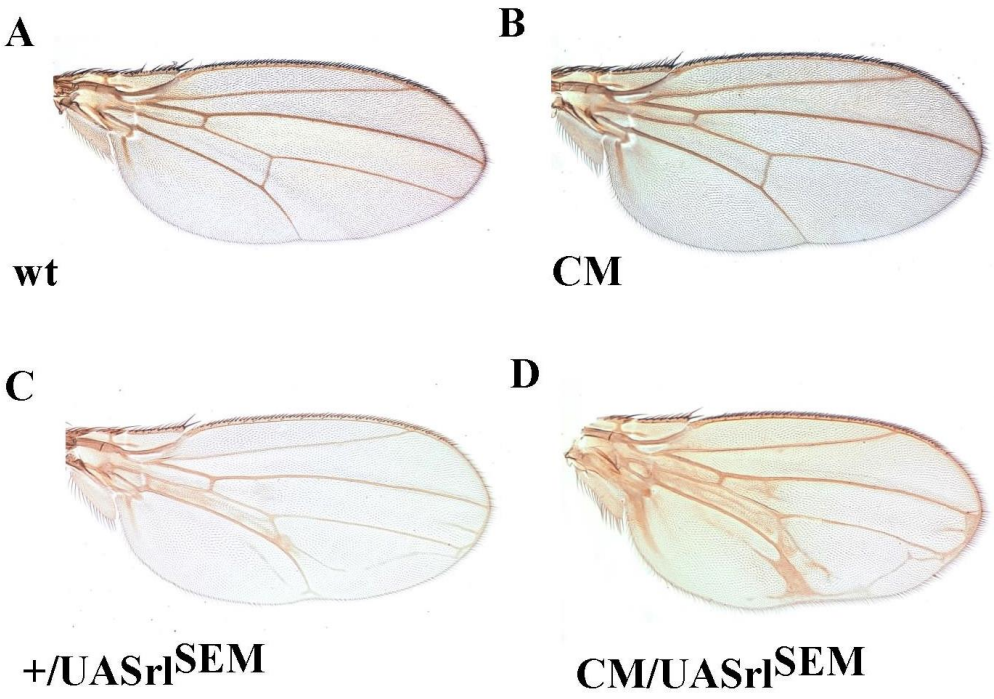

Figure 38: Wing phenotype as a result of EGFR/MAPK misexpression in wild-type and the *brk*^{CM}mutant

(A) Wild-type wing. (B) *brk*^{CM} wing appears morphologically wild-type. (C) Upregulation of EGFR/MAPK signaling in the posterior compartment of a wild-type disc (en-Gal4/UAS-rl^{SEM}) produces a mild defect in wing-vein patterning and some extra wing-veins. (D) Upregulation of EGFR/MAPK signaling in the posterior compartment of a *brk*^{CM} wing-disc results a more severe defect in the number and patterning of wing-veins compared to that in the wild-type (C).

4.0 MECHANISM OF BRINKER MEDIATED REPRESSION: INSIGHTS FROM BRINKER MUTANT ANALYSIS

4.1 DISCUSSION

4.1.1 Structure/Function analyses in *Drosophila*

A key approach facilitating the functional characterization of genes has been reverse genetics. This has been especially useful for characterizing newly uncovered genes such as those encoding for novel classes of noncoding RNAs that are otherwise not amenable to standard forward genetic approaches (Crown and Sekelsky, 2013). Structure-function analyses are more informative and more easily interpreted when modifications/mutations of the gene of interest are made in its endogenous copy, targeting any changes to its native locus to maintain chromosomal context. Gene targeting approaches for the modification of target loci has been routinely used in yeast (Rothstein, 1991) and a similar strategy has been adapted for use in mice where the targeted modification is selected in embryonic stem-cell culture and subsequently injected into blastocyst stage and descendants ultimately contribute to the germline (Thomas et al., 1986; Doetschman et al., 1988).

Two major strategies for *in vivo* gene-targeting introduced in *Drosophila* have been the ‘ends-in’ or insertional targeting (Rong and Golic, 2000) and ‘ends-out’ or replacement targeting

(Gong and Golic, 2003) approaches that both involve the introduction of the targeting cassette by P-element-mediated transgenesis followed by its excision and linearization *in vivo* creating a template for homologous recombination and then screening for the desired targeted integration. These strategies have been limited by being extremely labor-intensive because events occur at only low frequencies and the unpredictability of their success (O'Keefe et al., 2007; Crown and Sekelsky, 2013), so that although knockouts could be made, it was far too much work to envisage doing structure/function analyses which would involve utilizing this technique multiple times. However, more recently improvements to the technique have been made so that creating targeted modifications of the *Drosophila* genome have become much more efficient, although the gene-specific rates are still somewhat variable (Huang et al., 2008; Huang et al., 2009) and still probably too low to contemplate the repeated targeting involved in structure/function analyses. However, targeted struction/function analyses have become realistic by combining these gene-targeting techniques with ØC31 integrase mediated transgenesis.

Here I have performed a structure/function analysis of the transcriptional repressor, Brk, by replacing the endogenous *brk* gene with a ØC31 bacteriophage *attP* site into which mutant forms of *brk* were integrated by integrase-mediated transgenesis (Huang et al., 2009). This genomic engineering approach is described in detail earlier [see chapter 2.0] and has been very successful. This success has been aided by the prior knowledge that a *brk* knockout would be lethal and that the gene is on the X chromosome and generating the initial knockout was far less labor intensive than expected. Subsequent integration of modified forms into the knockout was straightforward but was also made easier by the *brk* transcription unit being small and lacking introns. Further since genomic engineering also utilizes minimal *attP* and *attB* sites, 50 and 53 bp respectively (Groth et al., 2000) the final engineered alleles are identical to the endogenous

gene except for a minimal hybrid *attR* site in the 5' UTR and a loxP site in the 3'UTR (Huang et al., 2009). However, while overall the genomic engineering approach used in the present study has been far less work than expected, in particular generating the initial knockout, each *brk* mutant when generated carried the *white* marker and other extra sequences that had to be removed by *loxP*-Cre mediated site-specific recombination to ensure that the mutant *brk* locus was identical to the wild-type *brk* gene with the exception of the desired mutations, and doing this for every *brk* mutant engineered followed by their validation turned out be more work than anticipated. Taking this into consideration the success and relative ease of gene targeting demonstrated in the present study is encouraging and may be considered for other structure/function analyses in *Drosophila.* Below I will discuss other alternative strategies and novelties in gene targeting approaches that have been introduced.

An approach very similar to genomic engineering has been introduced by Gao *et. al.* (2008) in which a gene is replaced by an *attP* site but appears slightly more time-consuming than genomic engineering. This site-specific integrase mediated repeated targeting (SIRT) technique combined 'ends in' targeting with the ØC31 integrase system (Gao et al., 2008) but it produces a tandem duplication with every insertion event at the host locus which then needs to be reduced to a single copy via a round of double strand break and repair by single strand annealing. This does, however, remove the marker gene and is essentially equivalent to the use of Cre/loxP to remove the white marker in the genomic engineering technique I employed. The latter is straightforward, while the reduction step in SIRT is achieved by random recombination and will generate several possible outcomes that need to be screened through to identify the desired reduction (Gao et al., 2008). The advantage of SIRT is that it does not leave behind a *loxP* site at the targeted locus.

The perceived problem with genomic engineering is generating the initial knockout, and has led to some new approaches that allow direct modification of genomic DNA without the need to utilize the knock out technology (Bateman et al., 2013; Wesolowska and Rong, 2013). Without going into too much detail, these approaches require the presence of one or in some cases two *attP* sites within about 70 kb of a gene of interest (the *attP* sites having been previously introduced into the genome by random P-element mediated transgenesis), into which is introduced a modified region of the adjacent genome, producing a tandem duplication that can be reduced to a single locus, with some of the reductions carrying the desired modification. It is unclear how feasible these approaches will be and they are likely to involve extensive recombineering of large bacterial artificial chromosomes (BACs) to generate the initial genomic modification, so structure/function analyses will require repeated rounds of these procedures and are likely be labor intensive.

Another approach that does not involve the initial step of generating a knockout is the use of rescuing genomic constructs carrying modifications, which are introduced into a null mutant background and so provide the only genetic activity. This approach is dependent on identifying a region of genomic DNA that can fully substitute for the endogenous gene and integrating it into the genome (in any location that does not influence activity). This has become more feasible with ØC31 integrase technology, which allows the integration of relatively large DNA molecules such as BACs (>200 kb), which will likely include the transcription unit and regulatory elements of a gene of interest. Even assuming a rescuing fragment can be identified, modification of the BACs is not trivial and all subsequent experiments have to be done with the rescuing constructs in a mutant background.

In conclusion, the approach I have taken has been very successful and although it was a significant amount of work it answered the questions I originally posed, and it is unlikely that any of the alternative approaches would have been any less trivial.

4.1.2 Brk uses Gro as its primary co-repressor but CtBP and 3R are required in some tissues

Our goal was to generate mutations that disrupted the ability of Brk to recruit the co-repressor Gro and CtBP and/or delete the less well characterized 3R repression domain and test their activity in different tissues at different times of development to determine if and why Brk protein needs these three different ways to repress transcription. Previous studies with Brk and other transcription factors that can recruit both co-repressors indicated that Gro recruitment is essential for at least some of the activities of these transcription factors [see section 1.7 and 1.9] but the reason for recruiting CtBP has proved more elusive. This study has confirmed that Gro recruitment is essential for Brk activity, but has also shown that Brk needs to recruit CtBP and possesses the 3R domain for full activity in some tissues, in particular during oogenesis. Why Brk requires CtBP and 3R in addition to Gro has been discussed below with reference to the hypotheses proposed quantitative, qualitative, noise reduction and availability.

4.1.3 Availability: the key reason why Brk cannot rely on Gro

Lethality of the brk^{GM} mutant reveals Gro recruitment to be necessary for Brk activity. The $brk^{\Delta 3RCM}$ mutant, which utilizes Gro as its sole repressive activity, can progress from fertilization to an almost morphologically wild-type adult indicating Gro is just about sufficient in this

regard, at least for some individuals, but this is not always the case as $brk^{\Delta 3RCM}$ mutants often die as embryos and eggs laid by females containing $brk^{\Delta 3RCM}$ clones in follicle cells have aberrant egg-shell pattering, characteristic of *brk* null mutants (Fig. 32D,E), indicating Brk needs 3R and/or CtBP to provide full activity. The single mutants, $brk^{\Delta 3R}$ and brk^{CM}, also show less severe egg-shell defects and reduced fertility, the latter probably relating to a defective micropyle, the structure through which sperm normally enter (Fig. 32F,G). The apparent inactivity of $Brk^{\Delta 3RCM}$ protein in follicle cells appears to be explained by levels of active unphosphorylated Gro being reduced here. The egg-shell is patterned by the oocyte surrounding follicle cells where Brk is expressed at high levels in the dorsal anterior (Fig. 35A). This coincides with high levels of EGFR signaling (Fig. 35B) and previous studies have shown that Gro activity is attenuated following phosphorylation by EGFR signaling (Hasson et al., 2005; Cinnamon et al., 2008). As expected, lower levels of unphosphorylated or active Gro are found in the dorsal-anterior follicle cells (Fig. 35C). Consistent with the activity of $Brk^{\Delta 3RCM}$ being compromised by EGFR-dependent downregulation of Gro activity, upregulation of EGFR signaling in the wing disc of brk^{CM} mutants results in derepression of the targets, salE1 and ombZ (Fig. 37).

EGFR signaling also probably reduces the levels of active Gro available for Brk in other tissues, including the larval ventral ectoderm where Brk activity is required to ensure proper patterning of the denticle belts and where EGFR signaling is known play a key role (Sanson, 2001). Many $brk^{\Delta 3RCM}$ mutants do not survive embryogenesis and demonstrate defects in denticle patterning similar but weaker than null mutants (Fig. 27B,E). In addition, the ventral denticle belt phenotype of brk^{GM} mutants (Fig. 27D) is less severe than brk^{KO} or brk^{3M} mutants (Fig. 27B,C). Thus, CtBP and 3R together appear to provide repressive activity in the ventral ectoderm.

4.1.4 Quantitative: CtBP and 3R together provide more activity for Brk

Gro is sufficient for Brk to repress the embryonic targets tested in this study, so this does not support the notion that CtBP and/or 3R are there to boost Brk's repressive activity if Gro is available, at least in this situation. However, if Brk is unable to recruit Gro, either because its GiM has been mutated as in brk^{GM}, or because Gro is unavailable because its activity has been downregulated by Receptor Tyrosine Kinase (RTK) signaling, as in the follicle cells during oogenesis, then its activity appears to be dependent on both 3R and CtBP. As described above, these appear to function additively during oogenesis where together they are more efficient than when acting alone, so that eggs laid by mothers carrying $brk^{\Delta 3RCM}$ follicle cell clones have stronger egg-shell phenotypes than those laid by either brk^{CM} or $brk^{\Delta 3R}$ mothers (Fig. 32D-G).

4.1.5 Qualitative: only some targets are Gro-specific

The essential question here is whether any Brk targets specifically require Gro, CtBP or 3R for their repression. As mentioned, Gro is necessary and sufficient for repression of the early embryonic Brk targets *dpp*, *tld* and *zen*, as they are derepressed in brk^{GM} but not $brk^{\Delta 3RCM}$ embryos (Fig. 26D,E). However, this does not necessarily make these qualitatively 'Gro-specific' as CtBP activity may be too low at these stages. In fact, overexpression of Brk proteins unable to recruit Gro can repress *dpp* and *zen*, but not *tld* (Hasson et al., 2001), indicating *tld*, but not *dpp* nor *zen*, is such a Gro-specific target. Such specificity is presumably related to the distance over which the two co-repressors are known to function, with Gro repressing over a much longer distance (Cai et al., 1996; Dubnicoff et al., 1997; Martinez and Arnosti, 2008). In the wing disc, the targets *sal*, the salE1 reporter and ombZ are only partially derepressed in

brk^{GM} mutant clones (Fig. 23C,D and 18K), indicating they are not Gro-specific. In regard to repression of *sal* and salE1, there is a clear distinction between CtBP/Gro and 3R; Brk^{CMGM}, which has 3R as it sole repressive activity, is unable to repress *sal* (Fig. 23G,H) or salE1 (Fig. 18L), indicating that these targets are CtBP/Gro-specific, as indicated in earlier studies (Winter and Campbell, 2004). This is not a question of 3R possessing less activity as proteins lacking the CiM and GiM are unable to repress salE1 even if overexpressed at high levels, but can fully repress ombZ (Campbell unpublished). How 3R functions to repress transcription is currently not known so we cannot, as yet, explain this distinction between it and CtBP/Gro.

No Brk targets have been characterized in the follicle cells, but when identified it would be expected that these might be partially derepressed in both *brk*^{CM} and *brk*$^{\Delta 3R}$ mutants and possibly completely derepressed in *brk*$^{\Delta 3RCM}$ mutants based on the egg shell phenotypes; although there may be some differences between *brk*^{CM} and *brk*$^{\Delta 3R}$, given the differences between CtBP and 3R just discussed. However, again this would not imply that these targets are CtBP/3R-specific, because the inability of Gro to participate in their repression is presumed to be due to its unavailability. Thus, although studies have indicated that transcription factors that have the ability to recruit both Gro and CtBP they may only recruit one at specific targets (Bianchi-Frias et al., 2004), this may not reflect a co-repressor specificity for individual targets, but rather is an outcome of the availability of the co-repressors in cells in which the transcription factor regulate the targets.

4.1.6 Noise reduction: CtBP/3R may be important to reduce variability in some tissues

While Gro is sufficient for Brk to make a morphologically wild-type fly, it is unable to do so consistently every time, and although *brk*$^{\Delta 3RCM}$ survivors can appear wild-type they often possess

at least one defective wing (Fig. 20E and Fig. 21A,B). Thus, CtBP and 3R might be functioning to minimize stochastic variation and supplement Gro activity during wing development. It should be noted that EGFR signaling is occurring in the wing disc and may compromise Gro activity enough to put it at the threshold of being sufficient for Brk.

4.1.7 Implications of phosphorylation-dependent attenuation of Gro

It is possible that if Gro was available in all cells the CiM and 3R domain of Brk would be dispensable and so, at least for Brk, downregulation of Gro by RTK mediated phosphorylation could be considered inconvenient. This may be true for other transcription factors, including Hairy, Hairless and Knirps, which also function in multiple tissues many of which are exposed to RTK signaling and that may explain why these transcription factors need to resort to recruiting CtBP as well as Gro (Nagel and Preiss, 2011). It should also be noted that Gro activity can be downregulated in other ways including phosphorylation by Homeodomain-interacting protein kinase 2 (Choi et al., 2005). Also the poly(ADP-ribose) polymerase 1 (PARP1) mediates polyADP ribosylation and ejection of the mammalian ortholog of Gro, transducing-like Enhancer of split 1 (TLE1) proteins from promoters (Ju et al., 2004). In addition dominant-negative forms of TLE proteins have been identified in vertebrates that include the Amino-terminal Enhancer of Split (AES) proteins that antagonize the repressive activity of the TLE co-repressor proteins by multimerizing with them and disabling transcription factor binding or formation of productive repressive complexes at target promoters (Roose et al., 1998; Allen et al., 2006; Beagle and Johnson, 2010).

This downregulation of Gro activity has been explained in terms of reducing the activity of specific repressors in specific tissues, such as E(Spl) factors during wing vein formation

(Hasson et al., 2005; Orian et al., 2007). This appears to be a somewhat illogical way to downregulate activity of specific repressors as there are almost certainly many other transcription factors utilizing Gro in the same cells and in other tissues exposed to RTK signaling and their activity may be compromised. There is no data indicating whether the downregulation of Gro activity in follicle cells serves any purpose and could simply be a consequence of this regulatory mechanism functioning in other tissues. However, it has serious implications for Brk and has necessitated it to be versatile in its mechanisms of repression. The above scenario naively implies that Brk initially possessed a GiM, but phosphorylation of Gro by RTK signaling has necessitated Brk to acquire additional repression mechanisms, one of which was the acquisition of a CiM. The exact sequence of events in an evolutionary sense will probably be impossible to sort out. And the analysis of Brk from more primitive insects does not provide much insight. Brk from the more closely related insects, including Lepidopterans (butterflies), Coleopterans (beetles) and Hymenopterans (wasps) all have a CiM and GiM. Data from more distantly related insects is much sparser; this includes Brk from a human louse, *Pediculus* that has a divergent CiM and a putative GiM, and also from a Hemipteran (aphids), which seem to possess neither a GiM nor a CiM. Consequently re-evaluation of genome sequence data from more primitive insects is needed to answer if the CiM and the additional repression domain 3R have been acquired in *Drosophila* Brk as a result of Gro downregulation by phosphorylation.

Of course the possibility that downregulation of Gro activity does serve a purpose for Brk in the follicle cells cannot be ruled out; for example, if Gro were available here it might provide Brk with too much activity or allow it to inappropriately repress a target that CtBP or 3R cannot. The ideal experiment to test these possibilities would be to assess egg-shell phenotypes after

driving unphosphorylatable Gro at physiological levels in a *brk*$^{\Delta 3RCM}$ mutant, but currently this is technically challenging.

4.1.8 Co-repressor availability as a general explanation for versatility of repression mechanisms in transcription factors

The idea that repressors need to be versatile in their repressive mechanisms because of variable co-repressor availability presumably extends outside of Brk and Hairless, Hairy and Knirps. In fact, other repressors in *Drosophila* possess both CiMs and GiMs, including, for example, Snail (unpublished observations). This may not be simply related to downregulation of Gro activity as CtBP activity can be modulated also; for example the SUMOylation of mammalian CtBP1 is essential for its nuclear retention, the acetylation of CtBP2 by p300 and phosphorylation of CtBP1 by p21activated kinase modulates their nuclear retention and repressive activity (Lin et al., 2003; Zhao et al., 2006). Also the transducer β-like proteins, TBL1 and TBLR1 mediate ubiquitylation dependent degradation of mammalian CtBP1 and CtBP2 when recruited to NCoR and SMRT repressor complexes (Perissi et al., 2008). Similar to Gro and CtBP the availability of other co-repressors may also be modulated, for example the MAPK mediated phosphorylation leads to the nuclear export and inactivation of another co-repressor complex, SMRT (Hong and Privalsky, 2000).

In addition to covalent modifications, binding with ligands has also been demonstrated to influence the repressive activity of co-repressors. For example CtBP proteins that are homologous to NAD-dependent D hydroxyacid dehydrogenases (Kumar et al., 2002; Nardini et al., 2003) require NAD binding for mediating repression (Sutrias-Grau and Arnosti, 2004), thus

enabling them to sense the redox environment of the cell and elicit concomitant changes in gene expression in response (Zhang, Q. et al., 2002; Zhang et al., 2007).

Also many co-repressors in *Drosophila* and vertebrates are differentially spliced and in many cases the resultant isoforms exhibit developmental stage and timing specific activity (Sharma et al., 2008).

Thus, an important consideration raised by the present study is that care should be taken in assuming a transcription factor requires and can use a specific co-repressor to repress its targets in a particular tissue simply because it possesses an interaction motif for it.

4.2 CONCLUSIONS

This study provides the first physiologically relevant insight into why any transcription factor recruits multiple co-repressors and possesses additional repression domains in a multicellular organism. I have dissected the requirement of CtBP, Gro and 3R for full Brk activity in all tissues where it is known to function. At physiological levels Gro alone is required and largely sufficient for most Brk activity except in certain contexts such as in the ventral ectoderm of the late embryo and in oogenesis where its repressive activity is downregulated. In these situations CtBP and 3R are required to achieve full Brk activity as Gro is incapacitated. CtBP and 3R also appear to act in concert to minimize noise, thus supplementing Gro activity to ensure consistency in Brk activity during development and patterning.

The results of this study also indicate that in many situations Brk activity is dependent on Gro simply because CtBP and 3R probably are unable to provide high enough activity. Further utilization of CtBP and 3R to supplement Brk activity in oogenesis because its primary co-

repressor Gro is not available there provides insight to why several transcription factors require more than one repression domain or co-repressor for their activity. It brings to light that in addition to the possibility of differential requirement for target regulation, co-repressor availability and activity may also be extensively modulated, providing additional layers of regulation, at which cellular cues maybe integrated with changes in transcriptional output and alterations in cell-fate and physiology.

4.3 FUTURE DIRECTIONS

4.3.1 Test whether Brinker can recruit CtBP and Gro simultaneously and similar tests of co-repressor binding to mutant proteins

Chromatin immunoprecipitation (ChIP) and co-immunoprecipitation (Co-IP) approaches can be used on wing imaginal discs and embryos in order to test directly if in the wild-type Brk is actually recruited to its targets in the wing disc and embryo. To test whether Brk in turn recruits both CtBP and Gro simultaneously at its target loci, ChIP and Co-IP studies may be performed with embryos and wing imaginal discs in *brk* mutants where CiM and GiM are mutated individually and in combination. It is expected that if CtBP and Gro are recruited simultaneously at some or all Brk target genes, they will not be present in Brk containing complexes at target genes in brk^{CMGM} mutants; CtBP but not Gro will be bound in Brk complexes at target genes in brk^{GM} mutants and Gro but not CtBP will be bound in Brk complexes at target genes in brk^{CM} mutants. To aid these analyses endogenous TAP/FLAG- and HA-tagged wild-type *brk* rescue and HA tagged endogenous mutant *brk* strains with CiM and GiM mutated individually or in

combination have already been created in the course of this study. Further tagged forms of CtBP and Gro maybe used to test if they are present at target sites bound by Brk if antibodies prove unreliable.

An alternate approach would be to follow the binding of endogenous Brk to specific sites in salivary gland polytene chromosomes followed by testing if both Gro and CtBP are also associated with these sites simultaneously. For this purpose GFP and RFP tagged forms of wild-type *brk* rescue mutants that have already been generated.

4.3.2 Delineation of minimal Brinker protein

Previous work from our lab and the findings of the current study indicate the Gro is the primary co-repressor for Brk although it is not completely sufficient on its own, thus additional studies will need to be carried out to determine the minimal Brk protein that can rescue the brk^{KO} mutant. A key observation behind these experiments is that other studies in our lab have revealed that a Brk protein possessing a single GiM does not behave in a manner expected from some of the results presented here when it is overexpressed in the wing. In particular, it fails to repress salE1; full repression of this reporter requiring GiM plus additional sequences outside of the defined GiM. These studies could be extended to the endogenous gene. This would involve generating additional constructs containing the GiM plus other regions of the Brk protein so as to define the minimal protein capable of rescuing the brk^{KO}.

4.3.3 Analysis of a Brinker activity in spermatogenesis

The present study has also uncovered a potentially novel role of Brk in *Drosophila* spermatogenesis as 69% $brk^{\Delta 3RCM}$ males are found to be infertile. While Brk has not been previously implicated in regulating spermatogenesis, the BMP ligand Dpp has ben shown to play an important role in the maintenance of the germline stem cell (GSC) niche (Lim et al., 2012). GSCs unable to respond to BMP signaling ectopically express the differentiation gene, *bag of marbles* (*bam)* and undergo premature differentiation (Lim et al., 2012). Hence, it will be interesting to evaluate if Brk is expressed and required as a negative regulator of Dpp signaling in the course of *Drosophila* spermatogenesis. To this end, first Brk expression, if any, in the testes maybe evaluated by *in situ* hybridization and antibody staining. If Brk is expressed in wild-type testes, mutant *brk* testes can be assessed to identify defects, for example the deregulation of *bam.* If this is successful and a role for Brk in spermatogenesis is validated additional studies can be carried out using the endogenous *brk* mutants generated in this study to identify whether there is a differential requirement for Gro, CtBP and 3R in mediating Brk function in spermatogenesis.

4.3.4 Direct testing of whether Gro is sufficient for Brk activity in the ovary if it is not phosphorylated

Results from the current study indicate that Gro is unavailable for Brk in the follicle cells of the ovary, and that this requires Brk to be able to recruit CtBP and possess the 3R domain. This poses the question of whether Brk could do without its CiM and 3R if Gro was available. To test this, one could drive an unphosphorylatable form of Gro in the follicle cells at the same time as

generating $brk^{\Delta 3RCM}$ mutant clones. The key to this experiment is to drive Gro at physiological levels and this may prove more difficult than might be perceived as all reported experiments with driving Gro indicate it has dominant effects. Also, being unable to downregulate Gro activity may have pleiotropic effects, with other repressors now being able to recruit it, and so it is unclear what might happen. An alternative is to directly fuse an unphosphorylatable form of Gro to a Brk protein lacking its repression domains and replace the endogenous *brk* gene with this to determine if it can fully rescue without the need to utilize CtBP or 3R.

4.3.5 Repeat approach with other repressors

It would be interesting, but obviously a significant amount of work, to repeat the approach described here with the other repressors known to recruit both CtBP and Gro, namely Hairy, Hairless and Knirps. This would directly test the proposal that these factors recruit CtBP largely because Gro is unavailable in some tissues.

5.0 MATERIALS AND METHODS

5.1 FLY STRAINS UTILIZED FOR STUDY

Flies carrying the following existing alleles or transgenes were used: *brk*^{F124}, *brk*^{E427}, *brk*^{F138}, *brk*^{M68}, *brk*^{XA}, *gro*^{E48}, *gro*^{MB36}, *CtBP*$^{l(3)87De\text{-}10}$, *gro* RNAi (P{TRiP.HMS01506}attP2), hs-GFP (*Avic\GFPhs.T:Hsap\MYC*), hs-flp (P{hsp70-flp}1, P{hsp70-flp}3 and P{ry[+t7.2]=hsFLP}86E), FRT18A (P{ry[+t7.2]=neoFRT}18A), FRT82B (P{neoFRT}82B), arm-lacZ (P{w[+mC]=arm-lacZ.V}MM1), omb-lacZ (P{lacW}biPol-1), *w*^{1118}, *y*^{1}, Ras RNAi - Ras85D (P{TRiP.JF02478}attP2), FM7c, *y*^{1} *arm*^{4} *w**/FM7c,P{Ftz/lacC}YH1 (see Bloomington stock BL616), FM7d, w^{1} oc^{1} ptg^{1}/C(1)DX, y^{1} f^{1} (BL5269), P{w[+mC]=Crew}DH2), *vasaØC31*$^{ZH\text{-}102D}$ (M{vas-int.B}ZH-102D), UAS-GFP (P{UAS-GFP.T:Myc.T:nls2}1), tub>CD2>Gal4 GAL4$^{Scer\backslash FRT.Rnor\backslash CD2.\alpha Tub84B}$), hs-Cre (P{Crew}DH2), hs hid, hs-iSceI (see BL25680), Gal4-221[w-] (P{?GawB}221w-), UAS-rl^{SEM} (rl$^{Sem.Scer\backslash UAS}$), en-Gal4 (P{en2.4-GAL4}e16E). All genotypes are as denoted in FlyBase (http://flybase.bio.indiana.edu), where more information on each can be found. *brk*^{XGXM2} is a previously unpublished viable allele generated by mobilization of a P-element insertion, *brk*^{XG} (Campbell, unpublished).

The salE1 reporter is a 471 bp fragment at the 3'end of the sal1.8S/E fragment of (Kuhnlein et al., 1997) cloned into a GFP reporter containing vector, pHSB, which is a modified version of pH-Stinger (Barolo et al., 2000) in which two Brk binding sites in the hsp70 promoter

have been mutated.

5.2 GENERATION OF BRINKER KNOCKOUT STRAIN

This was as carried out as described in (Huang et al., 2008; Huang et al., 2009) and (Zhou et al., 2012). Genomic fragments flanking the *brk* coding region and extending into the 5' and 3' UTR were amplified by PCR and cloned into the P-element vector, pGX-PCM1 (identical to pGX-attP-WN with the absence of the SphI site (Zhou et al., 2012), resulting in a targeting construct that positions a ØC31 *attP* site and *white-neo* marker flanked by *loxP* sites between the genomic fragments and having an FRT site, I-SceI site and UAS-reaper outside. w^+ transformants were generated by P-element-mediated transgenesis and a line on the third chromosome was selected to use to make the knockout (*brk* is on the X). Males were crossed to the 6934-hid strain of (Huang et al., 2008) that carries Y-linked hs-hid (to facilitate virgin collection), hs-Flp and hs-I-SceI; progeny were heat-shocked to induce expression of the latter and flp-out and then linearize DNA from the integrated targeted construct to form a template for homologous recombination. These progeny, potentially carrying a recombination event in their germ cells, were initially crossed to the ubiquitous driver Gal4221[w-] to induce expression of UAS-Rpr and thus kill any progeny carrying non-targeted integrations of the targeting construct construct as well as any progeny carrying the original targeting construct on the third (it was not found necessary to use G418 to select for the *white-neo* marker). 15,000 progeny were screened for w^+ females (*brk* mutants are lethal) yielding six hundred potential candidates. Out of these ten failed to produce w^+ males and all mapped to the X. They were initially tested by crossing to a viable brk^{XGXM2} hypomorphic allele and all failed to complement indicating they carried a *brk* mutant and were

potential knockouts. These were further validated molecularly as described in detail above. Following validation of a w^+ knockout, the w^+ marker was removed by crossing to a hs-Cre line and this was further validated molecularly.

5.3 GENERATION OF BRINKER MUTANTS

In vitro generated *brk* mutants contained within UAS constructs have been described previously, these are CtBP interaction motif (CiM) from PMDLSLG to AMAAALA as a *Not*I site, the GiM from FKPY to FAAA and created a *No*tI site, 3R region (deletion of residues 148-200 replace with a *Bgl*II site (RS) (Winter and Campbell, 2004). These were cloned into the pGE-attB^{GMR} vector (Huang et al., 2009) that includes a øC31 *attB* site, injected into the $brk^{KO\text{-}w\text{-}}$ strain expressing øC31 integrase (*vasa-øC31*) and integrations identified as w^+ transformants. These were validated molecularly and the w^+ marker was removed by crossing to a hs-Cre line. They were then revalidated molecularly.

5.4 ANALYSIS OF PROTEIN LEVELS IN BRINKER MUTANTS

Brk protein levels in mutant cells were compared to that in wild-type by antibody staining wing discs containing mutant clones. After ensuring the confocal detectors were not saturated, clones were chosen for analysis in the lateral-most regions of the disc (to eliminate any effects from *brk* autorepression in more medial locations) and levels of fluorescence were averaged over the region of a clone using ImageJ software and compared to that for an adjacent wild-type twin-

spot. For every *brk* mutant, twenty independently generated clones were assessed and the average fluorescence level within a clone was measured along with the average within an adjacent wild-type twin spot and the relative difference was calculated; a relative value of 1 will then indicate no difference and a Chi-square test with trend was used to determine whether relative mutant Brk/wild-type levels were significantly different from this 'expected' value.

5.5 GENETIC MOSAICS, OVEREXPRESSION AND RNAI MEDIATED KNOCKDOWN IN FOLLICULAR EPITHELIUM

Loss of function clones of brk^{KO}, brk^{3M}, brk^{GM}, $brk^{\Delta 3RCM}$ and were generated by the FRT/FLP recombination technique (Xu and Rubin, 1993). Adult females were heat-shocked twice for 1 hour at 37°C with a 6-8 hour interval in between. Eggs were evaluated 5-8 days after heat-shock treatment. To ubiquitously knockdown and overexpress EGFR/Ras/MAPK signaling Ras85D RNAi and UAS-rl^{SEM}, respectively, were driven by CY2-Gal4, GR1-Gal4, tubG4 which drive ubiquitous Gal4 in follicle cells of stage 10 egg chambers. For each similar results were obtained with all three drivers.

5.6 CLONAL ANALYSIS, OVEREXPRESSION AND RNAI MEDIATED KNOCKDOWN IN WING DISC

Homozygous mutant clones were generated in imaginal discs by hsflp/FRT-induced mitotic recombination (Xu and Rubin, 1993). Clones were generated in the second or early third instar of

larvae with the following genotypes:

y omb-lacZ brk^{KO} FRT18A/hsGFP FRT18A; hs-flp (and similarly for other *brk* mutants). Clones were identified by the loss of GFP.

y omb-lacZ brk^{GM} FRT18A/arm-lacZ FRT18A; salE1; hs-flp (and similarly for brk^{CMGM}). Clones were identified by loss of ß-gal staining.

hs-flp; FRT82B $CtBP^{l(3)87De\text{-}10}$ gro^{E48}/FRT82B arm-lacZ (and the same for single mutant clones). Clones were identified by loss of ß-gal staining.

UAS-rl^{SEM} was overexpressed with en-Gal4 (expressed in the posterior compartment of the wing-disc). Gro was knocked down by RNAi using en-Gal4.

5.7 RNA IN SITU HYBRIDIZATION, IMMUNOHISTOCHEMISTRY AND ANALYSIS OF WINGS

In situ hybridizations on 2-4 hour-old embryos were carried out as previously described (Tautz and Pfeifle, 1989) using digoxigenin labeled probes against *dpp*, *tld* and *zen*. *Brk* mutants were balanced over FM7c-FtzlacZ and hemizygous embryos for every genotype were selected by absence of *lacZ* expression detected using a fluorescein labeled lacZ probe and anti-fluorescein antibody (Roche). Dissection and staining of wing discs were carried out according to standard techniques. Antibodies used were: anti-Sal (rabbit, 1:50; (Kuhnlein et al., 1994) anti-βgal (rabbit, 1:2000, Cappell; and chicken, 1:2000, Abcam), anti-Brk (1:400; (Campbell and Tomlinson, 1999), monoclonal anti-Gro antibody (1:2000; Developmental Studies Hybridoma bank). Adult fly wings were mounted in GMM (Lawrence and Johnston, 1986).

5.8 CUTICLE PREPARATION

Unhatched larvae (24-48 hours old) were dechorionated in 50% bleach, transferred into clearing medium (acetic acid: glycerol, 4:1) for 30 minutes at 65°C and then at room temperature for at least a day prior to mounting in CMCP-10 mounting medium (Polysciences): lactic acid, 3:1.

5.9 FEMALE FERTILITY ANALYSIS

Female fertility was evaluated by mating one hundred 3-4 day-old females of the appropriate genotypes to an equal number of 2-3 day-old w^{1118} males in a cage. The grape-juice plate of the cage was supplied with fresh yeast and changed twice a day. At 8-10 days from the day of initial mating, unfertilized eggs were scored by the absence of nuclei from 5-6 hr DAPI-stained embryos. For every genotype indicated three independent experiments were carried out with at least 100 eggs scored in each.

5.10 MALE FERTILITY ANALYSIS

Male fertility was evaluated by mating individual 3-day-old males of the indicated genotypes to three w^{1118} females. After 4 days, each vial was visually examined to ensure eggs had been laid and the parents were discarded. After 12 days (of initial mating) the vials were visually inspected to determine if the eggs laid had hatched into larva. Crosses where no eggs were laid or those laid that did not hatch into larva were scored to determine the number of infertile males. The

number of infertile males was determined from four independent experiments with 50 males of each genotype tested in every experiment.

5.11 IMAGING AND STATISTICAL ANALYSIS

Confocal imaging was performed on Olympus Fluoview FV1000 confocal laser scanning microscope. Differential interference contrast and light microscopy were performed with Nikon Eclipse E800 microscope (SPOT 5.1 basic software). Images were analyzed using ImageJ software. Images were minimally processed and resampled to 300 dpi using Adobe Photoshop. All data shown are mean±s_e_m. Statistical analysis was carried out using Graphpad Prism 6.0 software and statistical significance was tested using Mann Whitney U test, the Kruskal Wallis multiple comparison test or Chi-square test for trend as indicated.

APPENDIX A

(I) Primers and constructs used to generate endogenous Brk mutants

Construct no./name	Primer name	Primer Sequence
PL2037/brkKOattPMi	brkExtF2	atgcggtaccCAAGTCAAGATGGCTTGC
	3SB5PH2	gatcggtaccTCATAACTCGCGATCTGG
PL2045/brk3PKOF1R2E	brk3PKO1F1	gatccctaggATGCGCCTATACATAGAG
	brk3PKOR2	gatccctaggGTGTTCGTGTCAATGTGTGC

PL2038/brkKOattPM: generated from brkKOattPMi and brk3PKPF1R2E to make brk^{KO}

PL2544/brkKORNE	brkKORFN	gatcgaattcAAGCGCAAGCGTCGCACGC
	brkKORR	gatcggtaccTTAATATTTCCCTTGGCAATG

(II) Primer sequences for validation of Brk mutants

brkres5P2: gatcCAGCATTTTGATATAAATTTATC

attRrev2: gatcGTTACCCCAGTTGGGGCACTAC

wneo3F3: CTGTTTATTGCCCCCTCAAA

brkres3P1: gatcCGCGTGCGTGTATATTTATG

5brkfor2: gatcGTGCCAGTGTGTGTATGTG

brkres5P1: gatcGAATGCTCAAGAGACGTG

brkwrev1: gatcGAGGGAGAGTCACAAAACG

brkAmpF2: gatcCTGGTGAGTACTCAACCAAG

3Pbrkrev1: gatcGTATAGGCGCATTCCTAGGC

attLfor1: gatcCTCTCAGTTGGGGGCGTAG

brk3PR3: GCCCTATGTTTTGCCCAGT

5brkfor1: gatcCACAACTATATAGATTTGAAAC

loxPR3: GAAGTTATGGTACCTTAATATTTC

(III) List of constructs generated

Construct no./name

PL2545/brkKORGN: wt brk (control) that rescues brk^{KO} functionally and molecularly. Generated by cloning brkKORNE into pGE-attB-GMR vector.

PL3049/3PFKORGN: wt brk (control) with a C-terminal 2X HA tag generated from PL3049/3PF3GPSNN (cloned from preexisting constructs) and brkKORGN.

PL2404/brkCMG: CiM mutated. Generated from brkCMN3S (already present) and brkKORGN.

PL2405/brkCMFG: CiM mutated and C-terminal end tagged with 2X HA. Cloned from 3PFKORGN and PL2446/brkCMN3S (preexisting construct). Not injected.

PL2410/brkNAG or brkΔ3R brkKORFN gatcgaattcAAGCGCAAGCGTCGCACGC
brkE1R gatcagatctCTCCTTCTGATGCTGCAT

PL2411/brkNAFG: $brk^{\Delta 3R}$ 3R domain deleted and tagged at C-terminus with 2X HA. Generated from brkNAG and PL3048/3PF3GPSNN. Not injected.

PL2412/brkNACMG or brkΔ3RCM: The CiM mutated and 3R domain deleted. Generated by cloning PD2438/brkNAKOE1E (preexisting construct) into brkCMG.

PL2413/brkNACMFG: CiM mutated, 3R domain deleted and tagged at C-terminus with 2X HA. Not injected.

PL2406/brkGMG: The GiM mutated. Generated from brkKORGN and PL2017/brkNAGMNU (preexisting construct). Not injected due to unwanted mutations.

PL2407/brkGMFG: GiM mutated and tagged at C-terminus with 2X HA. Generated from 3PFKORGN and PL2017/brkNAGMNU. Not used due to unwanted mutations.

PL3044/brkGMGNN: GiM mutated. Generated from brkGMG and PL2955/brkCMGMGN.

PL3045/brkGMFGNN: GiM mutated and tagged at C-terminus with 2X HA. Generated from brkGMFG and 3PFKORGN. Not injected.

PL2408/brkCMGMG: CiM and GiM mutated. Generated from brkCMGM and brkKORGN.

PL2017/brkNAGMNU (preexisting construct). Not injected due to unwanted mutations.

PL2409/brkCMGMFG: CiM and GiM mutated and tagged at C-terminus with 2X HA. Not used due to unwanted mutations.

PL2955/brkCMGMGN: CiM and GiM mutated. Generated from brkCMGMG and brkKORGN.

PL3046/brkCMGMFGNN: CiM, GiM mutated and tagged at C-terminus with 2X HA. Generated from brkCMGMFG and 3PFKORGN. Not injected.

PL2414/brkNAGMG or brkΔ3RGM: GiM mutated and 3R deleted. Generated from brkGMG and PD2438/brkNAKOE1E (preexisting PCR DNA). Not injected due to unwanted mutations.

PL2415/brkNAGMFG: GiM mutated, 3R deleted and tagged at C-terminus with 2X HA. Generated from brkNAFG and PL2017/brkNAGMNU. Not used due to unwanted mutations.

PL2957/brkNAGMGN or brkΔ3RGM: GiM mutated and 3R deleted. Generated from brkNAGMG and brkKORGN.

PL3047/brkNAGMFGNN: GiM mutated, 3R domain deleted and tagged at C-terminus with 2X HA. Generated from brkNAGMFG and 3PFKORGN. Not injected.

PL2416/brk3MG: CiM, GiM mutated and 3R deleted. Generated from brkCMGMG and PD2438/brkNAKOE1E (preexisting PCR DNA). Not injected due to unwanted mutations.

PL2417/brk3MFG: CiM, GiM mutated and 3R deleted and tagged at the C-terminus with 2X HA. Generated from brkNAFG and brkCMGMFG. Not used due to unwanted mutations.

PL2959/brk3MGN: CiM, GiM mutated and 3R domain deleted. Generated from brk3MG and brkKORGN.

PL2076/brk3MFGNN: CiM, GiM mutated and 3R deleted and tagged at the C-terminus with 2X HA. Generated from brk3MFG and 3PFKORGN.

APPENDIX B

Primers used to *brk* and *shn* riboprobes

Construct	Primer name	Primer Sequence
brkribo	brkFor	ATGGATAGCAGCAGCGAACAGTTG
	brkRev	TTAGGCCACCAGGGTCAGGTTTGTG
shnribo	shnF1	GACAGAAAGAGCACGAAGCAGCC
	shnR1	TTGCTTGCCGCTGCTGAAGCAGCC

BIBLIOGRAPHY

Affolter, M. and Basler, K. (2007). The Decapentaplegic morphogen gradient: from pattern formation to growth regulation. *Nature reviews. Genetics* **8**, 663-674.

Ahmad, K. F., Melnick, A., Lax, S., Bouchard, D., Liu, J., Kiang, C. L., Mayer, S., Takahashi, S., Licht, J. D. and Prive, G. G. (2003). Mechanism of SMRT corepressor recruitment by the BCL6 BTB domain. *Molecular cell* **12**, 1551-1564.

Ahn, J. W., Lee, Y. A., Ahn, J. H. and Choi, C. Y. (2009). Covalent conjugation of Groucho with SUMO-1 modulates its corepressor activity. *Biochemical and biophysical research communications* **379**, 160-165.

Akam, M. (1989). Drosophila development: making stripes inelegantly. *Nature* **341**, 282-283.

Akimaru, H., Chen, Y., Dai, P., Hou, D. X., Nonaka, M., Smolik, S. M., Armstrong, S., Goodman, R. H. and Ishii, S. (1997). Drosophila CBP is a co-activator of cubitus interruptus in hedgehog signalling. *Nature* **386**, 735-738.

Alexandre, C., Lecourtois, M. and Vincent, J. (1999). Wingless and Hedgehog pattern Drosophila denticle belts by regulating the production of short-range signals. *Development* **126**, 5689-5698.

Allen, T., van Tuyl, M., Iyengar, P., Jothy, S., Post, M., Tsao, M. S. and Lobe, C. G. (2006). Grg1 acts as a lung-specific oncogene in a transgenic mouse model. *Cancer research* **66**, 1294-1301.

Amleh, A., Nair, S. J., Sun, J., Sutherland, A., Hasty, P. and Li, R. (2009). Mouse cofactor of BRCA1 (Cobra1) is required for early embryogenesis. *PloS one* **4**, e5034.

Ansari, A. Z., Bradner, J. E. and O'Halloran, T. V. (1995). DNA-bend modulation in a repressor-to-activator switching mechanism. *Nature* **374**, 371-375.

Armstrong, J. A., Papoulas, O., Daubresse, G., Sperling, A. S., Lis, J. T., Scott, M. P. and Tamkun, J. W. (2002). The Drosophila BRM complex facilitates global transcription by RNA polymerase II. *The EMBO journal* **21**, 5245-5254.

Arnone, M. I. and Davidson, E. H. (1997). The hardwiring of development: organization and function of genomic regulatory systems. *Development* **124**, 1851-1864.

Arnosti, D. N. and Kulkarni, M. M. (2005). Transcriptional enhancers: Intelligent enhanceosomes or flexible billboards? *Journal of cellular biochemistry* **94**, 890-898.

Arnosti, D. N., Gray, S., Barolo, S., Zhou, J. and Levine, M. (1996). The gap protein knirps mediates both quenching and direct repression in the Drosophila embryo. *The EMBO journal* **15**, 3659-3666.

Atchison, L., Ghias, A., Wilkinson, F., Bonini, N. and Atchison, M. L. (2003). Transcription factor YY1 functions as a PcG protein in vivo. *The EMBO journal* **22**, 1347-1358.
Bach, I., Rodriguez-Esteban, C., Carriere, C., Bhushan, A., Krones, A., Rose, D. W., Glass, C. K., Andersen, B., Izpisua Belmonte, J. C. and Rosenfeld, M. G. (1999). RLIM inhibits functional activity of LIM homeodomain transcription factors via recruitment of the histone deacetylase complex. *Nature genetics* **22**, 394-399.
Bajoghli, B. (2007). Evolution of the Groucho/Tle gene family: gene organization and duplication events. *Development genes and evolution* **217**, 613-618.
Ballas, N., Battaglioli, E., Atouf, F., Andres, M. E., Chenoweth, J., Anderson, M. E., Burger, C., Moniwa, M., Davie, J. R., Bowers, W. J. et al. (2001). Regulation of neuronal traits by a novel transcriptional complex. *Neuron* **31**, 353-365.
Barolo, S. and Levine, M. (1997). hairy mediates dominant repression in the Drosophila embryo. *The EMBO journal* **16**, 2883-2891.
Barolo, S. and Posakony, J. W. (2002). Three habits of highly effective signaling pathways: principles of transcriptional control by developmental cell signaling. *Genes & development* **16**, 1167-1181.
Barolo, S., Carver, L. A. and Posakony, J. W. (2000). GFP and beta-galactosidase transformation vectors for promoter/enhancer analysis in Drosophila. *Biotechniques* **29**, 726, 728, 730, 732.
Barolo, S., Stone, T., Bang, A. G. and Posakony, J. W. (2002). Default repression and Notch signaling: Hairless acts as an adaptor to recruit the corepressors Groucho and dCtBP to Suppressor of Hairless. *Genes & development* **16**, 1964-1976.
Barrio, R. and de Celis, J. F. (2004). Regulation of spalt expression in the Drosophila wing blade in response to the Decapentaplegic signaling pathway. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 6021-6026.
Bastock, R. and St Johnston, D. (2008). Drosophila oogenesis. *Current biology : CB* **18**, R1082-1087.
Basu, A. and Atchison, M. L. (2010). CtBP levels control intergenic transcripts, PHO/YY1 DNA binding, and PcG recruitment to DNA. *Journal of cellular biochemistry* **110**, 62-69.
Bateman, J. R., Palopoli, M. F., Dale, S. T., Stauffer, J. E., Shah, A. L., Johnson, J. E., Walsh, C. W., Flaten, H. and Parsons, C. M. (2013). Captured segment exchange: a strategy for custom engineering large genomic regions in Drosophila melanogaster. *Genetics* **193**, 421-430.
Beagle, B. and Johnson, G. V. (2010). AES/GRG5: more than just a dominant-negative TLE/GRG family member. *Developmental dynamics : an official publication of the American Association of Anatomists* **239**, 2795-2805.
Beamer, L. J. and Pabo, C. O. (1992). Refined 1.8 A crystal structure of the lambda repressor-operator complex. *Journal of molecular biology* **227**, 177-196.
Becalska, A. N. and Gavis, E. R. (2009). Lighting up mRNA localization in Drosophila oogenesis. *Development* **136**, 2493-2503.
Bejsovec, A. and Martinez Arias, A. (1991). Roles of wingless in patterning the larval epidermis of Drosophila. *Development* **113**, 471-485.
Berg, C. A. (2005). The Drosophila shell game: patterning genes and morphological change. *Trends in genetics : TIG* **21**, 346-355.

Bertrand-Burggraf, E., Hurstel, S., Daune, M. and Schnarr, M. (1987). Promoter properties and negative regulation of the uvrA gene by the LexA repressor and its amino-terminal DNA binding domain. *Journal of molecular biology* **193**, 293-302.
Bhaumik, S. R., Raha, T., Aiello, D. P. and Green, M. R. (2004). In vivo target of a transcriptional activator revealed by fluorescence resonance energy transfer. *Genes & development* **18**, 333-343.
Bianchi-Frias, D., Orian, A., Delrow, J. J., Vazquez, J., Rosales-Nieves, A. E. and Parkhurst, S. M. (2004). Hairy transcriptional repression targets and cofactor recruitment in Drosophila. *PLoS biology* **2**, E178.
Birrane, G., Soni, A. and Ladias, J. A. (2009). Structural basis for DNA recognition by the human PAX3 homeodomain. *Biochemistry* **48**, 1148-1155.
Bischof, J., Maeda, R. K., Hediger, M., Karch, F. and Basler, K. (2007). An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 3312-3317.
Blair, S. S. (1995). Compartments and appendage development in Drosophila. *Bioessays* **17**, 299-309.
Boehm, A. K., Saunders, A., Werner, J. and Lis, J. T. (2003). Transcription factor and polymerase recruitment, modification, and movement on dhsp70 in vivo in the minutes following heat shock. *Molecular and cellular biology* **23**, 7628-7637.
Boettiger, A. N. and Levine, M. (2009). Synchronous and stochastic patterns of gene activation in the Drosophila embryo. *Science* **325**, 471-473.
Boyd, J. M., Subramanian, T., Schaeper, U., La Regina, M., Bayley, S. and Chinnadurai, G. (1993). A region in the C-terminus of adenovirus 2/5 E1a protein is required for association with a cellular phosphoprotein and important for the negative modulation of T24-ras mediated transformation, tumorigenesis and metastasis. *The EMBO journal* **12**, 469-478.
Boyer, L. A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L. A., Lee, T. I., Levine, S. S., Wernig, M., Tajonar, A., Ray, M. K. et al. (2006). Polycomb complexes repress developmental regulators in murine embryonic stem cells. *Nature* **441**, 349-353.
Bracken, A. P., Dietrich, N., Pasini, D., Hansen, K. H. and Helin, K. (2006). Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. *Genes & development* **20**, 1123-1136.
Breen, T. R. and Duncan, I. M. (1986). Maternal expression of genes that regulate the bithorax complex of Drosophila melanogaster. *Developmental biology* **118**, 442-456.
Browning, D. F. and Busby, S. J. (2004). The regulation of bacterial transcription initiation. *Nature reviews. Microbiology* **2**, 57-65.
Brummel, T. J., Twombly, V., Marques, G., Wrana, J. L., Newfeld, S. J., Attisano, L., Massague, J., O'Connor, M. B. and Gelbart, W. M. (1994). Characterization and relationship of Dpp receptors encoded by the saxophone and thick veins genes in Drosophila. *Cell* **78**, 251-261.
Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J. and Greenberg, M. E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857-868.
Bryant, P. J. (1970). Cell lineage relationships in the imaginal wing disc of Drosophila melanogaster. *Developmental biology* **22**, 389-411.

Buscarlet, M., Hermann, R., Lo, R., Tang, Y., Joachim, K. and Stifani, S. (2009). Cofactor-activated phosphorylation is required for inhibition of cortical neuron differentiation by Groucho/TLE1. *PloS one* **4**, e8107.
Cai, H. N., Arnosti, D. N. and Levine, M. (1996). Long-range repression in the Drosophila embryo. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 9309-9314.
Campbell, G. and Tomlinson, A. (1999). Transducing the Dpp morphogen gradient in the wing of Drosophila: regulation of Dpp targets by brinker. *Cell* **96**, 553-562.
Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R. S. and Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* **298**, 1039-1043.
Carter, D., Chakalova, L., Osborne, C. S., Dai, Y. F. and Fraser, P. (2002). Long-range chromatin regulatory interactions in vivo. *Nature genetics* **32**, 623-626.
Chakrabarti, S. R., Sood, R., Nandi, S. and Nucifora, G. (2000). Posttranslational modification of TEL and TEL/AML1 by SUMO-1 and cell-cycle-dependent assembly into nuclear bodies. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 13281-13285.
Chen, G., Nguyen, P. H. and Courey, A. J. (1998). A role for Groucho tetramerization in transcriptional repression. *Molecular and cellular biology* **18**, 7259-7268.
Chen, G., Fernandez, J., Mische, S. and Courey, A. J. (1999). A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in Drosophila development. *Genes & development* **13**, 2218-2230.
Chen, J. D. and Evans, R. M. (1995). A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature* **377**, 454-457.
Chen, Y. and Schupbach, T. (2006). The role of brinker in eggshell patterning. *Mechanisms of development* **123**, 395-406.
Chinnadurai, G. (2007). Transcriptional regulation by C-terminal binding proteins. *Int J Biochem Cell Biol* **39**, 1593-1607.
Choi, C. Y., Kim, Y. H., Kim, Y. O., Park, S. J., Kim, E. A., Riemenschneider, W., Gajewski, K., Schulz, R. A. and Kim, Y. (2005). Phosphorylation by the DHIPK2 protein kinase modulates the corepressor activity of Groucho. *The Journal of biological chemistry* **280**, 21427-21436.
Chopra, V. S., Kong, N. and Levine, M. (2012). Transcriptional repression via antilooping in the Drosophila embryo. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 9460-9464.
Cinnamon, E. and Paroush, Z. (2008). Context-dependent regulation of Groucho/TLE-mediated repression. *Current opinion in genetics & development* **18**, 435-440.
Cinnamon, E., Helman, A., Ben-Haroush Schyr, R., Orian, A., Jimenez, G. and Paroush, Z. (2008). Multiple RTK pathways downregulate Groucho-mediated repression in Drosophila embryogenesis. *Development* **135**, 829-837.
Clapier, C. R. and Cairns, B. R. (2009). The biology of chromatin remodeling complexes. *Annu Rev Biochem* **78**, 273-304.
Cohen, S. M. (1993). Imaginal Disc Development. In *The Development of Drosophila melanogaster* (eds M. Bate and A. Martinez Arias). Plainview, N.Y.: Cold Spring Harbor Laboratory Press.

Cordier, F., Hartmann, B., Rogowski, M., Affolter, M. and Grzesiek, S. (2006). DNA recognition by the brinker repressor--an extreme case of coupling between binding and folding. *Journal of molecular biology* **361**, 659-672.
Cowley, D. O. and Graves, B. J. (2000). Phosphorylation represses Ets-1 DNA binding by reinforcing autoinhibition. *Genes & development* **14**, 366-376.
Crown, K. N. and Sekelsky, J. (2013). Targeted gene replacement in Drosophila goes the distance. *Genetics* **193**, 377-381.
Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A. and Pirrotta, V. (2002). Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. *Cell* **111**, 185-196.
Daniels, D. L. and Weis, W. I. (2005). Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. *Nature structural & molecular biology* **12**, 364-371.
Das, P., Maduzia, L. L., Wang, H., Finelli, A. L., Cho, S. H., Smith, M. M. and Padgett, R. W. (1998). The Drosophila gene Medea demonstrates the requirement for different classes of Smads in dpp signaling. *Development* **125**, 1519-1528.
Dearolf, C. R., Topol, J. and Parker, C. S. (1989). The caudal gene product is a direct activator of fushi tarazu transcription during Drosophila embryogenesis. *Nature* **341**, 340-343.
Degner, S. C., Verma-Gaur, J., Wong, T. P., Bossen, C., Iverson, G. M., Torkamani, A., Vettermann, C., Lin, Y. C., Ju, Z., Schulz, D. et al. (2011). CCCTC-binding factor (CTCF) and cohesin influence the genomic architecture of the Igh locus and antisense transcription in pro-B cells. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 9566-9571.
Dehni, G., Liu, Y., Husain, J. and Stifani, S. (1995). TLE expression correlates with mouse embryonic segmentation, neurogenesis, and epithelial determination. *Mechanisms of development* **53**, 369-381.
Dekker, J., Rippe, K., Dekker, M. and Kleckner, N. (2002). Capturing chromosome conformation. *Science* **295**, 1306-1311.
del Alamo Rodriguez, D., Terriente Felix, J. and Diaz-Benjumea, F. J. (2004). The role of the T-box gene optomotor-blind in patterning the Drosophila wing. *Developmental biology* **268**, 481-492.
Delidakis, C., Preiss, A., Hartley, D. A. and Artavanis-Tsakonas, S. (1991). Two genetically and molecularly distinct functions involved in early neurogenesis reside within the Enhancer of split locus of Drosophila melanogaster. *Genetics* **129**, 803-823.
Doetschman, T., Maeda, N. and Smithies, O. (1988). Targeted mutation of the Hprt gene in mouse embryonic stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 8583-8587.
Dorman, J. B., James, K. E., Fraser, S. E., Kiehart, D. P. and Berg, C. A. (2004). bullwinkle is required for epithelial morphogenesis during Drosophila oogenesis. *Developmental biology* **267**, 320-341.
Doyen, C. M., An, W., Angelov, D., Bondarenko, V., Mietton, F., Studitsky, V. M., Hamiche, A., Roeder, R. G., Bouvet, P. and Dimitrov, S. (2006). Mechanism of polymerase II transcription repression by the histone variant macroH2A. *Molecular and cellular biology* **26**, 1156-1164.
Dressel, U., Thormeyer, D., Altincicek, B., Paululat, A., Eggert, M., Schneider, S., Tenbaum, S. P., Renkawitz, R. and Baniahmad, A. (1999). Alien, a highly conserved protein

with characteristics of a corepressor for members of the nuclear hormone receptor superfamily. *Molecular and cellular biology* **19**, 3383-3394.
Dubnicoff, T., Valentine, S. A., Chen, G., Shi, T., Lengyel, J. A., Paroush, Z. and Courey, A. J. (1997). Conversion of dorsal from an activator to a repressor by the global corepressor Groucho. *Genes & development* **11**, 2952-2957.
Ebright, R. H. (2000). RNA polymerase: structural similarities between bacterial RNA polymerase and eukaryotic RNA polymerase II. *Journal of molecular biology* **304**, 687-698.
Edwards, K. A. and Kiehart, D. P. (1996). Drosophila nonmuscle myosin II has multiple essential roles in imaginal disc and egg chamber morphogenesis. *Development* **122**, 1499-1511.
Enriquez, J., Boukhatmi, H., Dubois, L., Philippakis, A. A., Bulyk, M. L., Michelson, A. M., Crozatier, M. and Vincent, A. (2010). Multi-step control of muscle diversity by Hox proteins in the Drosophila embryo. *Development* **137**, 457-466.
Erkner, A., Roure, A., Charroux, B., Delaage, M., Holway, N., Core, N., Vola, C., Angelats, C., Pages, F., Fasano, L. et al. (2002). Grunge, related to human Atrophin-like proteins, has multiple functions in Drosophila development. *Development* **129**, 1119-1129.
Ernst, T., Chase, A. J., Score, J., Hidalgo-Curtis, C. E., Bryant, C., Jones, A. V., Waghorn, K., Zoi, K., Ross, F. M., Reiter, A. et al. (2010). Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders. *Nature genetics* **42**, 722-726.
Essers, M. A., Weijzen, S., de Vries-Smits, A. M., Saarloos, I., de Ruiter, N. D., Bos, J. L. and Burgering, B. M. (2004). FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. *The EMBO journal* **23**, 4802-4812.
Ezhkova, E., Pasolli, H. A., Parker, J. S., Stokes, N., Su, I. H., Hannon, G., Tarakhovsky, A. and Fuchs, E. (2009). Ezh2 orchestrates gene expression for the stepwise differentiation of tissue-specific stem cells. *Cell* **136**, 1122-1135.
Fanto, M., Clayton, L., Meredith, J., Hardiman, K., Charroux, B., Kerridge, S. and McNeill, H. (2003). The tumor-suppressor and cell adhesion molecule Fat controls planar polarity via physical interactions with Atrophin, a transcriptional co-repressor. *Development* **130**, 763-774.
Fischle, W., Wang, Y., Jacobs, S. A., Kim, Y., Allis, C. D. and Khorasanizadeh, S. (2003). Molecular basis for the discrimination of repressive methyl-lysine marks in histone H3 by Polycomb and HP1 chromodomains. *Genes & development* **17**, 1870-1881.
Fisher, A. L., Ohsako, S. and Caudy, M. (1996). The WRPW motif of the hairy-related basic helix-loop-helix repressor proteins acts as a 4-amino-acid transcription repression and protein-protein interaction domain. *Molecular and cellular biology* **16**, 2670-2677.
Fu, J., Gnatt, A. L., Bushnell, D. A., Jensen, G. J., Thompson, N. E., Burgess, R. R., David, P. R. and Kornberg, R. D. (1999). Yeast RNA polymerase II at 5 A resolution. *Cell* **98**, 799-810.
Gadbois, E. L., Chao, D. M., Reese, J. C., Green, M. R. and Young, R. A. (1997). Functional antagonism between RNA polymerase II holoenzyme and global negative regulator NC2 in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 3145-3150.
Gao, G., McMahon, C., Chen, J. and Rong, Y. S. (2008). A powerful method combining homologous recombination and site-specific recombination for targeted mutagenesis in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 13999-14004.
Garcia-Bellido, A., Ripoll, P. and Morata, G. (1973). Developmental compartmentalisation of the wing disk of Drosophila. *Nat New Biol* **245**, 251-253.

Garriga-Canut, M., Schoenike, B., Qazi, R., Bergendahl, K., Daley, T. J., Pfender, R. M., Morrison, J. F., Ockuly, J., Stafstrom, C., Sutula, T. et al. (2006). 2-Deoxy-D-glucose reduces epilepsy progression by NRSF-CtBP-dependent metabolic regulation of chromatin structure. *Nature neuroscience* **9**, 1382-1387.

Gaston, K. and Jayaraman, P. S. (2003). Transcriptional repression in eukaryotes: repressors and repression mechanisms. *Cell Mol Life Sci* **60**, 721-741.

Ge, K., Guermah, M., Yuan, C. X., Ito, M., Wallberg, A. E., Spiegelman, B. M. and Roeder, R. G. (2002). Transcription coactivator TRAP220 is required for PPAR gamma 2-stimulated adipogenesis. *Nature* **417**, 563-567.

Gerhart, J. (1999). 1998 Warkany lecture: signaling pathways in development. *Teratology* **60**, 226-239.

Geyer, P. K., Green, M. M. and Corces, V. G. (1990). Tissue-specific transcriptional enhancers may act in trans on the gene located in the homologous chromosome: the molecular basis of transvection in Drosophila. *The EMBO journal* **9**, 2247-2256.

Gilchrist, D. A., Dos Santos, G., Fargo, D. C., Xie, B., Gao, Y., Li, L. and Adelman, K. (2010). Pausing of RNA polymerase II disrupts DNA-specified nucleosome organization to enable precise gene regulation. *Cell* **143**, 540-551.

Gilchrist, D. A., Nechaev, S., Lee, C., Ghosh, S. K., Collins, J. B., Li, L., Gilmour, D. S. and Adelman, K. (2008). NELF-mediated stalling of Pol II can enhance gene expression by blocking promoter-proximal nucleosome assembly. *Genes & development* **22**, 1921-1933.

Gilchrist, D. A., Fromm, G., dos Santos, G., Pham, L. N., McDaniel, I. E., Burkholder, A., Fargo, D. C. and Adelman, K. (2012). Regulating the regulators: the pervasive effects of Pol II pausing on stimulus-responsive gene networks. *Genes & development* **26**, 933-944.

Goldstein, R. E., Cook, O., Dinur, T., Pisante, A., Karandikar, U. C., Bidwai, A. and Paroush, Z. (2005). An eh1-like motif in odd-skipped mediates recruitment of Groucho and repression in vivo. *Molecular and cellular biology* **25**, 10711-10720.

Golembo, M., Raz, E. and Shilo, B. Z. (1996). The Drosophila embryonic midline is the site of Spitz processing, and induces activation of the EGF receptor in the ventral ectoderm. *Development* **122**, 3363-3370.

Golic, K. G. and Lindquist, S. (1989). The FLP recombinase of yeast catalyzes site-specific recombination in the Drosophila genome. *Cell* **59**, 499-509.

Gong, W. J. and Golic, K. G. (2003). Ends-out, or replacement, gene targeting in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 2556-2561.

Goppelt, A., Stelzer, G., Lottspeich, F. and Meisterernst, M. (1996). A mechanism for repression of class II gene transcription through specific binding of NC2 to TBP-promoter complexes via heterodimeric histone fold domains. *The EMBO journal* **15**, 3105-3116.

Gray, S. and Levine, M. (1996). Transcriptional repression in development. *Current opinion in cell biology* **8**, 358-364.

Groth, A. C., Olivares, E. C., Thyagarajan, B. and Calos, M. P. (2000). A phage integrase directs efficient site-specific integration in human cells. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 5995-6000.

Groth, A. C., Fish, M., Nusse, R. and Calos, M. P. (2004). Construction of transgenic Drosophila by using the site-specific integrase from phage phiC31. *Genetics* **166**, 1775-1782.

Gu, J. Y., Park, J. M., Song, E. J., Mizuguchi, G., Yoon, J. H., Kim-Ha, J., Lee, K. J. and Kim, Y. J. (2002). Novel Mediator proteins of the small Mediator complex in Drosophila SL2 cells. *The Journal of biological chemistry* **277**, 27154-27161.

Guenther, M. G., Levine, S. S., Boyer, L. A., Jaenisch, R. and Young, R. A. (2007). A chromatin landmark and transcription initiation at most promoters in human cells. *Cell* **130**, 77-88.

Hakimi, M. A., Bochar, D. A., Chenoweth, J., Lane, W. S., Mandel, G. and Shiekhattar, R. (2002). A core-BRAF35 complex containing histone deacetylase mediates repression of neuronal-specific genes. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 7420-7425.

Hanna-Rose, W. and Hansen, U. (1996). Active repression mechanisms of eukaryotic transcription repressors. *Trends in genetics : TIG* **12**, 229-234.

Hasson, P., Muller, B., Basler, K. and Paroush, Z. (2001). Brinker requires two corepressors for maximal and versatile repression in Dpp signalling. *The EMBO journal* **20**, 5725-5736.

Hasson, P., Egoz, N., Winkler, C., Volohonsky, G., Jia, S., Dinur, T., Volk, T., Courey, A. J. and Paroush, Z. (2005). EGFR signaling attenuates Groucho-dependent repression to antagonize Notch transcriptional output. *Nature genetics* **37**, 101-105.

Hawley, D. K., Johnson, A. D. and McClure, W. R. (1985). Functional and physical characterization of transcription initiation complexes in the bacteriophage lambda OR region. *The Journal of biological chemistry* **260**, 8618-8626.

He, L., Wang, X. and Montell, D. J. (2011). Shining light on Drosophila oogenesis: live imaging of egg development. *Current opinion in genetics & development* **21**, 612-619.

He, X., Duque, T. S. and Sinha, S. (2012). Evolutionary origins of transcription factor binding site clusters. *Molecular biology and evolution* **29**, 1059-1070.

Heck, B. W., Zhang, B., Tong, X., Pan, Z., Deng, W. M. and Tsai, C. C. (2012). The transcriptional corepressor SMRTER influences both Notch and ecdysone signaling during Drosophila development. *Biology open* **1**, 182-196.

Heinzel, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W. M., Brard, G., Ngo, S. D., Davie, J. R. et al. (1997). A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. *Nature* **387**, 43-48.

Heltzel, A., Lee, I. W., Totis, P. A. and Summers, A. O. (1990). Activator-dependent preinduction binding of sigma-70 RNA polymerase at the metal-regulated mer promoter. *Biochemistry* **29**, 9572-9584.

Hoch, M., Gerwin, N., Taubert, H. and Jackle, H. (1992). Competition for overlapping sites in the regulatory region of the Drosophila gene Kruppel. *Science* **256**, 94-97.

Hong, S. H. and Privalsky, M. L. (2000). The SMRT corepressor is regulated by a MEK-1 kinase pathway: inhibition of corepressor function is associated with SMRT phosphorylation and nuclear export. *Molecular and cellular biology* **20**, 6612-6625.

Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C. K. et al. (1995). Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature* **377**, 397-404.

Horne-Badovinac, S. and Bilder, D. (2005). Mass transit: epithelial morphogenesis in the Drosophila egg chamber. *Developmental dynamics : an official publication of the American Association of Anatomists* **232**, 559-574.

Howard, K. and Ingham, P. (1986). Regulatory interactions between the segmentation genes fushi tarazu, hairy, and engrailed in the Drosophila blastoderm. *Cell* **44**, 949-957.

Hsieh, J. J., Zhou, S., Chen, L., Young, D. B. and Hayward, S. D. (1999). CIR, a corepressor linking the DNA binding factor CBF1 to the histone deacetylase complex. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 23-28.
Hu, X. and Lazar, M. A. (1999). The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors. *Nature* **402**, 93-96.
Huang, J., Zhou, W., Watson, A. M., Jan, Y. N. and Hong, Y. (2008). Efficient ends-out gene targeting in Drosophila. *Genetics* **180**, 703-707.
Huang, J., Zhou, W., Dong, W., Watson, A. M. and Hong, Y. (2009). From the Cover: Directed, efficient, and versatile modifications of the Drosophila genome by genomic engineering. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 8284-8289.
Huang, J. D., Schwyter, D. H., Shirokawa, J. M. and Courey, A. J. (1993). The interplay between multiple enhancer and silencer elements defines the pattern of decapentaplegic expression. *Genes & development* **7**, 694-704.
Hudson, J. B., Podos, S. D., Keith, K., Simpson, S. L. and Ferguson, E. L. (1998). The Drosophila Medea gene is required downstream of dpp and encodes a functional homolog of human Smad4. *Development* **125**, 1407-1420.
Humphrey, G. W., Wang, Y., Russanova, V. R., Hirai, T., Qin, J., Nakatani, Y. and Howard, B. H. (2001). Stable histone deacetylase complexes distinguished by the presence of SANT domain proteins CoREST/kiaa0071 and Mta-L1. *The Journal of biological chemistry* **276**, 6817-6824.
Inostroza, J. A., Mermelstein, F. H., Ha, I., Lane, W. S. and Reinberg, D. (1992). Dr1, a TATA-binding protein-associated phosphoprotein and inhibitor of class II gene transcription. *Cell* **70**, 477-489.
Ip, Y. T., Kraut, R., Levine, M. and Rushlow, C. A. (1991). The dorsal morphogen is a sequence-specific DNA-binding protein that interacts with a long-range repression element in Drosophila. *Cell* **64**, 439-446.
Ip, Y. T., Park, R. E., Kosman, D., Yazdanbakhsh, K. and Levine, M. (1992). dorsal-twist interactions establish snail expression in the presumptive mesoderm of the Drosophila embryo. *Genes & development* **6**, 1518-1530.
Jacobs, S. A. and Khorasanizadeh, S. (2002). Structure of HP1 chromodomain bound to a lysine 9-methylated histone H3 tail. *Science* **295**, 2080-2083.
Janssens, H., Hou, S., Jaeger, J., Kim, A. R., Myasnikova, E., Sharp, D. and Reinitz, J. (2006). Quantitative and predictive model of transcriptional control of the Drosophila melanogaster even skipped gene. *Nature genetics* **38**, 1159-1165.
Jayne, S., Zwartjes, C. G., van Schaik, F. M. and Timmers, H. T. (2006). Involvement of the SMRT/NCoR-HDAC3 complex in transcriptional repression by the CNOT2 subunit of the human Ccr4-Not complex. *The Biochemical journal* **398**, 461-467.
Jazwinska, A., Rushlow, C. and Roth, S. (1999a). The role of brinker in mediating the graded response to Dpp in early Drosophila embryos. *Development* **126**, 3323-3334.
Jazwinska, A., Kirov, N., Wieschaus, E., Roth, S. and Rushlow, C. (1999b). The Drosophila gene brinker reveals a novel mechanism of Dpp target gene regulation. *Cell* **96**, 563-573.
Jennings, B. H. and Ish-Horowicz, D. (2008). The Groucho/TLE/Grg family of transcriptional co-repressors. *Genome Biol* **9**, 205.
Jennings, B. H., Wainwright, S. M. and Ish-Horowicz, D. (2008). Differential in vivo requirements for oligomerization during Groucho-mediated repression. *EMBO reports* **9**, 76-83.

Jennings, B. H., Pickles, L. M., Wainwright, S. M., Roe, S. M., Pearl, L. H. and Ish-Horowicz, D. (2006). Molecular recognition of transcriptional repressor motifs by the WD domain of the Groucho/TLE corepressor. *Molecular cell* **22**, 645-655.
Jimenez, G., Paroush, Z. and Ish-Horowicz, D. (1997). Groucho acts as a corepressor for a subset of negative regulators, including Hairy and Engrailed. *Genes & development* **11**, 3072-3082.
Jones, W. D., Cayirlioglu, P., Kadow, I. G. and Vosshall, L. B. (2007). Two chemosensory receptors together mediate carbon dioxide detection in Drosophila. *Nature* **445**, 86-90.
Ju, B. G., Solum, D., Song, E. J., Lee, K. J., Rose, D. W., Glass, C. K. and Rosenfeld, M. G. (2004). Activating the PARP-1 sensor component of the groucho/ TLE1 corepressor complex mediates a CaMKinase IIdelta-dependent neurogenic gene activation pathway. *Cell* **119**, 815-829.
Junion, G., Spivakov, M., Girardot, C., Braun, M., Gustafson, E. H., Birney, E. and Furlong, E. E. (2012). A transcription factor collective defines cardiac cell fate and reflects lineage history. *Cell* **148**, 473-486.
Kagey, M. H., Newman, J. J., Bilodeau, S., Zhan, Y., Orlando, D. A., van Berkum, N. L., Ebmeier, C. C., Goossens, J., Rahl, P. B., Levine, S. S. et al. (2010). Mediator and cohesin connect gene expression and chromatin architecture. *Nature* **467**, 430-435.
Kamada, K., Shu, F., Chen, H., Malik, S., Stelzer, G., Roeder, R. G., Meisterernst, M. and Burley, S. K. (2001). Crystal structure of negative cofactor 2 recognizing the TBP-DNA transcription complex. *Cell* **106**, 71-81.
Kankel, M. W., Duncan, D. M. and Duncan, I. (2004). A screen for genes that interact with the Drosophila pair-rule segmentation gene fushi tarazu. *Genetics* **168**, 161-180.
Kao, H. Y., Downes, M., Ordentlich, P. and Evans, R. M. (2000). Isolation of a novel histone deacetylase reveals that class I and class II deacetylases promote SMRT-mediated repression. *Genes & development* **14**, 55-66.
Kato, Y., Kimoto, F., Susa, T., Nakayama, M., Ishikawa, A. and Kato, T. (2010). Pituitary homeodomain transcription factors HESX1 and PROP1 form a heterodimer on the inverted TAAT motif. *Molecular and cellular endocrinology* **315**, 168-173.
Kehle, J., Beuchle, D., Treuheit, S., Christen, B., Kennison, J. A., Bienz, M. and Muller, J. (1998). dMi-2, a hunchback-interacting protein that functions in polycomb repression. *Science* **282**, 1897-1900.
Keller, S. A., Mao, Y., Struffi, P., Margulies, C., Yurk, C. E., Anderson, A. R., Amey, R. L., Moore, S., Ebels, J. M., Foley, K. et al. (2000). dCtBP-dependent and -independent repression activities of the Drosophila Knirps protein. *Molecular and cellular biology* **20**, 7247-7258.
Kim, J. H., Cho, E. J., Kim, S. T. and Youn, H. D. (2005). CtBP represses p300-mediated transcriptional activation by direct association with its bromodomain. *Nature structural & molecular biology* **12**, 423-428.
Kim, S., Na, J. G., Hampsey, M. and Reinberg, D. (1997). The Dr1/DRAP1 heterodimer is a global repressor of transcription in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 820-825.
Kim, T. H., Barrera, L. O., Zheng, M., Qu, C., Singer, M. A., Richmond, T. A., Wu, Y., Green, R. D. and Ren, B. (2005). A high-resolution map of active promoters in the human genome. *Nature* **436**, 876-880.
Kim, Y. J., Cecchini, K. R. and Kim, T. H. (2011). Conserved, developmentally regulated mechanism couples chromosomal looping and heterochromatin barrier activity at the homeobox

gene A locus. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 7391-7396.
King, R. C. (1970). Ovarian development in Drosophila melanogaster. New York,: Academic Press.
Kirkpatrick, H., Johnson, K. and Laughon, A. (2001). Repression of dpp targets by binding of brinker to mad sites. *The Journal of biological chemistry* **276**, 18216-18222.
Kirov, N., Childs, S., O'Connor, M. and Rushlow, C. (1994). The Drosophila dorsal morphogen represses the tolloid gene by interacting with a silencer element. *Molecular and cellular biology* **14**, 713-722.
Kristjuhan, A., Walker, J., Suka, N., Grunstein, M., Roberts, D., Cairns, B. R. and Svejstrup, J. Q. (2002). Transcriptional inhibition of genes with severe histone h3 hypoacetylation in the coding region. *Molecular cell* **10**, 925-933.
Krummel, B. and Chamberlin, M. J. (1989). RNA chain initiation by Escherichia coli RNA polymerase. Structural transitions of the enzyme in early ternary complexes. *Biochemistry* **28**, 7829-7842.
Ku, M., Koche, R. P., Rheinbay, E., Mendenhall, E. M., Endoh, M., Mikkelsen, T. S., Presser, A., Nusbaum, C., Xie, X., Chi, A. S. et al. (2008). Genomewide analysis of PRC1 and PRC2 occupancy identifies two classes of bivalent domains. *PLoS genetics* **4**, e1000242.
Kuhnlein, R. P., Bronner, G., Taubert, H. and Schuh, R. (1997). Regulation of Drosophila spalt gene expression. *Mechanisms of development* **66**, 107-118.
Kuhnlein, R. P., Frommer, G., Friedrich, M., Gonzalez-Gaitan, M., Weber, A., Wagner-Bernholz, J. F., Gehring, W. J., Jackle, H. and Schuh, R. (1994). spalt encodes an evolutionarily conserved zinc finger protein of novel structure which provides homeotic gene function in the head and tail region of the Drosophila embryo. *The EMBO journal* **13**, 168-179.
Kumar, V., Carlson, J. E., Ohgi, K. A., Edwards, T. A., Rose, D. W., Escalante, C. R., Rosenfeld, M. G. and Aggarwal, A. K. (2002). Transcription corepressor CtBP is an NAD(+)-regulated dehydrogenase. *Molecular cell* **10**, 857-869.
Kunert, N., Wagner, E., Murawska, M., Klinker, H., Kremmer, E. and Brehm, A. (2009). dMec: a novel Mi-2 chromatin remodelling complex involved in transcriptional repression. *The EMBO journal* **28**, 533-544.
Lai, Z. C. and Rubin, G. M. (1992). Negative control of photoreceptor development in Drosophila by the product of the yan gene, an ETS domain protein. *Cell* **70**, 609-620.
Lammel, U. and Saumweber, H. (2000). X-linked loci of Drosophila melanogaster causing defects in the morphology of the embryonic salivary glands. *Development genes and evolution* **210**, 525-535.
Lammel, U., Meadows, L. and Saumweber, H. (2000). Analysis of Drosophila salivary gland, epidermis and CNS development suggests an additional function of brinker in anterior-posterior cell fate specification. *Mechanisms of development* **92**, 179-191.
Larsson, M. C., Domingos, A. I., Jones, W. D., Chiappe, M. E., Amrein, H. and Vosshall, L. B. (2004). Or83b encodes a broadly expressed odorant receptor essential for Drosophila olfaction. *Neuron* **43**, 703-714.
Lawrence, P. A. and Johnston, P. (1986). Methods of marking cells. Oxford Oxfordshire ; Washington, D.C.: IRL Press.
Lecuit, T., Brook, W. J., Ng, M., Calleja, M., Sun, H. and Cohen, S. M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the Drosophila wing. *Nature* **381**, 387-393.

Lee, C., Li, X., Hechmer, A., Eisen, M., Biggin, M. D., Venters, B. J., Jiang, C., Li, J., Pugh, B. F. and Gilmour, D. S. (2008). NELF and GAGA factor are linked to promoter-proximal pausing at many genes in Drosophila. *Molecular and cellular biology* **28**, 3290-3300.
Lee, J. R., Urban, S., Garvey, C. F. and Freeman, M. (2001). Regulated intracellular ligand transport and proteolysis control EGF signal activation in Drosophila. *Cell* **107**, 161-171.
Lee, T. I., Jenner, R. G., Boyer, L. A., Guenther, M. G., Levine, S. S., Kumar, R. M., Chevalier, B., Johnstone, S. E., Cole, M. F., Isono, K. et al. (2006). Control of developmental regulators by Polycomb in human embryonic stem cells. *Cell* **125**, 301-313.
Lee, Y. S. and Hwang, D. S. (1997). Occlusion of RNA polymerase by oligomerization of DnaA protein over the dnaA promoter of Escherichia coli. *The Journal of biological chemistry* **272**, 83-88.
Leon, C. and Lobe, C. G. (1997). Grg3, a murine Groucho-related gene, is expressed in the developing nervous system and in mesenchyme-induced epithelial structures. *Developmental dynamics : an official publication of the American Association of Anatomists* **208**, 11-24.
Letsou, A., Arora, K., Wrana, J. L., Simin, K., Twombly, V., Jamal, J., Staehling-Hampton, K., Hoffmann, F. M., Gelbart, W. M., Massague, J. et al. (1995). Drosophila Dpp signaling is mediated by the punt gene product: a dual ligand-binding type II receptor of the TGF beta receptor family. *Cell* **80**, 899-908.
Lewis, E. B. (1978). A gene complex controlling segmentation in Drosophila. *Nature* **276**, 565-570.
Lewis, M. (1996). Response: DNA Looping and Lac Repressor--CAP Interaction. *Science* **274**, 1931-1932.
Li, G. and Reinberg, D. (2011). Chromatin higher-order structures and gene regulation. *Current opinion in genetics & development* **21**, 175-186.
Li, L. M. and Arnosti, D. N. (2011). Long- and short-range transcriptional repressors induce distinct chromatin states on repressed genes. *Current biology : CB* **21**, 406-412.
Li, X. Y., MacArthur, S., Bourgon, R., Nix, D., Pollard, D. A., Iyer, V. N., Hechmer, A., Simirenko, L., Stapleton, M., Luengo Hendriks, C. L. et al. (2008). Transcription factors bind thousands of active and inactive regions in the Drosophila blastoderm. *PLoS biology* **6**, e27.
Liang, G., Lin, J. C., Wei, V., Yoo, C., Cheng, J. C., Nguyen, C. T., Weisenberger, D. J., Egger, G., Takai, D., Gonzales, F. A. et al. (2004). Distinct localization of histone H3 acetylation and H3-K4 methylation to the transcription start sites in the human genome. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 7357-7362.
Lim, C., Tarayrah, L. and Chen, X. (2012). Transcriptional regulation during Drosophila spermatogenesis. *Spermatogenesis* **2**, 158-166.
Lin, X., Sun, B., Liang, M., Liang, Y. Y., Gast, A., Hildebrand, J., Brunicardi, F. C., Melchior, F. and Feng, X. H. (2003). Opposed regulation of corepressor CtBP by SUMOylation and PDZ binding. *Molecular cell* **11**, 1389-1396.
Liu, C. L., Kaplan, T., Kim, M., Buratowski, S., Schreiber, S. L., Friedman, N. and Rando, O. J. (2005). Single-nucleosome mapping of histone modifications in S. cerevisiae. *PLoS biology* **3**, e328.
Liu, X., Sun, Y., Weinberg, R. A. and Lodish, H. F. (2001). Ski/Sno and TGF-beta signaling. *Cytokine & growth factor reviews* **12**, 1-8.
Lomvardas, S., Barnea, G., Pisapia, D. J., Mendelsohn, M., Kirkland, J. and Axel, R. (2006). Interchromosomal interactions and olfactory receptor choice. *Cell* **126**, 403-413.

Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F. and Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* **389**, 251-260.
Lund, A. H. and van Lohuizen, M. (2004). Polycomb complexes and silencing mechanisms. *Current opinion in cell biology* **16**, 239-246.
Lunyak, V. V., Burgess, R., Prefontaine, G. G., Nelson, C., Sze, S. H., Chenoweth, J., Schwartz, P., Pevzner, P. A., Glass, C., Mandel, G. et al. (2002). Corepressor-dependent silencing of chromosomal regions encoding neuronal genes. *Science* **298**, 1747-1752.
Lutterbach, B., Westendorf, J. J., Linggi, B., Patten, A., Moniwa, M., Davie, J. R., Huynh, K. D., Bardwell, V. J., Lavinsky, R. M., Rosenfeld, M. G. et al. (1998). ETO, a target of t(8;21) in acute leukemia, interacts with the N-CoR and mSin3 corepressors. *Molecular and cellular biology* **18**, 7176-7184.
Manavathi, B., Singh, K. and Kumar, R. (2007). MTA family of coregulators in nuclear receptor biology and pathology. *Nuclear receptor signaling* **5**, e010.
Manoli, D. S., Foss, M., Villella, A., Taylor, B. J., Hall, J. C. and Baker, B. S. (2005). Male-specific fruitless specifies the neural substrates of Drosophila courtship behaviour. *Nature* **436**, 395-400.
Marcal, N., Patel, H., Dong, Z., Belanger-Jasmin, S., Hoffman, B., Helgason, C. D., Dang, J. and Stifani, S. (2005). Antagonistic effects of Grg6 and Groucho/TLE on the transcription repression activity of brain factor 1/FoxG1 and cortical neuron differentiation. *Molecular and cellular biology* **25**, 10916-10929.
Margolis, J. and Spradling, A. (1995). Identification and behavior of epithelial stem cells in the Drosophila ovary. *Development* **121**, 3797-3807.
Marshall, N. F. and Price, D. H. (1992). Control of formation of two distinct classes of RNA polymerase II elongation complexes. *Molecular and cellular biology* **12**, 2078-2090.
Marshall, N. F. and Price, D. H. (1995). Purification of P-TEFb, a transcription factor required for the transition into productive elongation. *The Journal of biological chemistry* **270**, 12335-12338.
Martinez, C. A. and Arnosti, D. N. (2008). Spreading of a corepressor linked to action of long-range repressor hairy. *Molecular and cellular biology* **28**, 2792-2802.
Marty, T., Muller, B., Basler, K. and Affolter, M. (2000). Schnurri mediates Dpp-dependent repression of brinker transcription. *Nat Cell Biol* **2**, 745-749.
Mathieu, E. L., Finkernagel, F., Murawska, M., Scharfe, M., Jarek, M. and Brehm, A. (2012). Recruitment of the ATP-dependent chromatin remodeler dMi-2 to the transcribed region of active heat shock genes. *Nucleic acids research* **40**, 4879-4891.
Merabet, E. and Ackers, G. K. (1995). Calorimetric analysis of lambda cI repressor binding to DNA operator sites. *Biochemistry* **34**, 8554-8563.
Minami, M., Kinoshita, N., Kamoshida, Y., Tanimoto, H. and Tabata, T. (1999). brinker is a target of Dpp in Drosophila that negatively regulates Dpp-dependent genes. *Nature* **398**, 242-246.
Molenaar, M., Brian, E., Roose, J., Clevers, H. and Destree, O. (2000). Differential expression of the Groucho-related genes 4 and 5 during early development of Xenopus laevis. *Mechanisms of development* **91**, 311-315.
Monsalve, M., Mencia, M., Rojo, F. and Salas, M. (1996). Activation and repression of transcription at two different phage phi29 promoters are mediated by interaction of the same residues of regulatory protein p4 with RNA polymerase. *The EMBO journal* **15**, 383-391.

Moser, M. and Campbell, G. (2005). Generating and interpreting the Brinker gradient in the Drosophila wing. *Developmental biology* **286**, 647-658.
Moyle-Heyrman, G., Viswanathan, R., Widom, J. and Auble, D. T. (2012). Two-step mechanism for modifier of transcription 1 (Mot1) enzyme-catalyzed displacement of TATA-binding protein (TBP) from DNA. *The Journal of biological chemistry* **287**, 9002-9012.
Muller, B., Hartmann, B., Pyrowolakis, G., Affolter, M. and Basler, K. (2003). Conversion of an extracellular Dpp/BMP morphogen gradient into an inverse transcriptional gradient. *Cell* **113**, 221-233.
Muller, J., Hart, C. M., Francis, N. J., Vargas, M. L., Sengupta, A., Wild, B., Miller, E. L., O'Connor, M. B., Kingston, R. E. and Simon, J. A. (2002). Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. *Cell* **111**, 197-208.
Murawska, M., Hassler, M., Renkawitz-Pohl, R., Ladurner, A. and Brehm, A. (2011). Stress-induced PARP activation mediates recruitment of Drosophila Mi-2 to promote heat shock gene expression. *PLoS genetics* **7**, e1002206.
Murawsky, C. M., Brehm, A., Badenhorst, P., Lowe, N., Becker, P. B. and Travers, A. A. (2001). Tramtrack69 interacts with the dMi-2 subunit of the Drosophila NuRD chromatin remodelling complex. *EMBO reports* **2**, 1089-1094.
Murrell, A., Heeson, S. and Reik, W. (2004). Interaction between differentially methylated regions partitions the imprinted genes Igf2 and H19 into parent-specific chromatin loops. *Nature genetics* **36**, 889-893.
Muse, G. W., Gilchrist, D. A., Nechaev, S., Shah, R., Parker, J. S., Grissom, S. F., Zeitlinger, J. and Adelman, K. (2007). RNA polymerase is poised for activation across the genome. *Nature genetics* **39**, 1507-1511.
Nagel, A. C. and Preiss, A. (2011). Fine tuning of Notch signaling by differential co-repressor recruitment during eye development of Drosophila. *Hereditas* **148**, 77-84.
Nagel, A. C., Krejci, A., Tenin, G., Bravo-Patino, A., Bray, S., Maier, D. and Preiss, A. (2005). Hairless-mediated repression of notch target genes requires the combined activity of Groucho and CtBP corepressors. *Molecular and cellular biology* **25**, 10433-10441.
Nagy, L., Kao, H. Y., Chakravarti, D., Lin, R. J., Hassig, C. A., Ayer, D. E., Schreiber, S. L. and Evans, R. M. (1997). Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell* **89**, 373-380.
Nardini, M., Spano, S., Cericola, C., Pesce, A., Massaro, A., Millo, E., Luini, A., Corda, D. and Bolognesi, M. (2003). CtBP/BARS: a dual-function protein involved in transcription co-repression and Golgi membrane fission. *The EMBO journal* **22**, 3122-3130.
Nativio, R., Wendt, K. S., Ito, Y., Huddleston, J. E., Uribe-Lewis, S., Woodfine, K., Krueger, C., Reik, W., Peters, J. M. and Murrell, A. (2009). Cohesin is required for higher-order chromatin conformation at the imprinted IGF2-H19 locus. *PLoS genetics* **5**, e1000739.
Negre, N., Hennetin, J., Sun, L. V., Lavrov, S., Bellis, M., White, K. P. and Cavalli, G. (2006). Chromosomal distribution of PcG proteins during Drosophila development. *PLoS biology* **4**, e170.
Nellen, D., Burke, R., Struhl, G. and Basler, K. (1996). Direct and long-range action of a DPP morphogen gradient. *Cell* **85**, 357-368.
Newfeld, S. J., Chartoff, E. H., Graff, J. M., Melton, D. A. and Gelbart, W. M. (1996). Mothers against dpp encodes a conserved cytoplasmic protein required in DPP/TGF-beta responsive cells. *Development* **122**, 2099-2108.

Nibu, Y., Zhang, H. and Levine, M. (1998a). Interaction of short-range repressors with Drosophila CtBP in the embryo. *Science* **280**, 101-104.
Nibu, Y., Zhang, H., Bajor, E., Barolo, S., Small, S. and Levine, M. (1998b). dCtBP mediates transcriptional repression by Knirps, Kruppel and Snail in the Drosophila embryo. *The EMBO journal* **17**, 7009-7020.
Ntziachristos, P., Tsirigos, A., Van Vlierberghe, P., Nedjic, J., Trimarchi, T., Flaherty, M. S., Ferres-Marco, D., da Ros, V., Tang, Z., Siegle, J. et al. (2012). Genetic inactivation of the polycomb repressive complex 2 in T cell acute lymphoblastic leukemia. *Nature medicine* **18**, 298-301.
Nuthall, H. N., Husain, J., McLarren, K. W. and Stifani, S. (2002). Role for Hes1-induced phosphorylation in Groucho-mediated transcriptional repression. *Molecular and cellular biology* **22**, 389-399.
O'Keefe, L. V., Smibert, P., Colella, A., Chataway, T. K., Saint, R. and Richards, R. I. (2007). Know thy fly. *Trends in genetics : TIG* **23**, 238-242.
Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H. and Nakatani, Y. (1996). The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87**, 953-959.
Orian, A., Delrow, J. J., Rosales Nieves, A. E., Abed, M., Metzger, D., Paroush, Z., Eisenman, R. N. and Parkhurst, S. M. (2007). A Myc-Groucho complex integrates EGF and Notch signaling to regulate neural development. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 15771-15776.
Oswald, F., Kostezka, U., Astrahantseff, K., Bourteele, S., Dillinger, K., Zechner, U., Ludwig, L., Wilda, M., Hameister, H., Knochel, W. et al. (2002). SHARP is a novel component of the Notch/RBP-Jkappa signalling pathway. *The EMBO journal* **21**, 5417-5426.
Pagan, J. K., Arnold, J., Hanchard, K. J., Kumar, R., Bruno, T., Jones, M. J., Richard, D. J., Forrest, A., Spurdle, A., Verdin, E. et al. (2007). A novel corepressor, BCoR-L1, represses transcription through an interaction with CtBP. *The Journal of biological chemistry* **282**, 15248-15257.
Pankratz, M. J., Seifert, E., Gerwin, N., Billi, B., Nauber, U. and Jackle, H. (1990). Gradients of Kruppel and knirps gene products direct pair-rule gene stripe patterning in the posterior region of the Drosophila embryo. *Cell* **61**, 309-317.
Park, J. M., Gim, B. S., Kim, J. M., Yoon, J. H., Kim, H. S., Kang, J. G. and Kim, Y. J. (2001). Drosophila Mediator complex is broadly utilized by diverse gene-specific transcription factors at different types of core promoters. *Molecular and cellular biology* **21**, 2312-2323.
Paroush, Z., Finley, R. L., Jr., Kidd, T., Wainwright, S. M., Ingham, P. W., Brent, R. and Ish-Horowicz, D. (1994). Groucho is required for Drosophila neurogenesis, segmentation, and sex determination and interacts directly with hairy-related bHLH proteins. *Cell* **79**, 805-815.
Payankaulam, S. and Arnosti, D. N. (2009). Groucho corepressor functions as a cofactor for the Knirps short-range transcriptional repressor. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 17314-17319.
Payankaulam, S., Li, L. M. and Arnosti, D. N. (2010). Transcriptional repression: conserved and evolved features. *Current biology : CB* **20**, R764-771.
Penton, A. and Hoffmann, F. M. (1996). Decapentaplegic restricts the domain of wingless during Drosophila limb patterning. *Nature* **382**, 162-164.
Peri, F. and Roth, S. (2000). Combined activities of Gurken and decapentaplegic specify dorsal chorion structures of the Drosophila egg. *Development* **127**, 841-850.

Perissi, V., Scafoglio, C., Zhang, J., Ohgi, K. A., Rose, D. W., Glass, C. K. and Rosenfeld, M. G. (2008). TBL1 and TBLR1 phosphorylation on regulated gene promoters overcomes dual CtBP and NCoR/SMRT transcriptional repression checkpoints. *Molecular cell* **29**, 755-766.

Perissi, V., Staszewski, L. M., McInerney, E. M., Kurokawa, R., Krones, A., Rose, D. W., Lambert, M. H., Milburn, M. V., Glass, C. K. and Rosenfeld, M. G. (1999). Molecular determinants of nuclear receptor-corepressor interaction. *Genes & development* **13**, 3198-3208.

Peterlin, B. M. and Price, D. H. (2006). Controlling the elongation phase of transcription with P-TEFb. *Molecular cell* **23**, 297-305.

Picard, F., Kurtev, M., Chung, N., Topark-Ngarm, A., Senawong, T., Machado De Oliveira, R., Leid, M., McBurney, M. W. and Guarente, L. (2004). Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. *Nature* **429**, 771-776.

Pile, L. A. and Wassarman, D. A. (2000). Chromosomal localization links the SIN3-RPD3 complex to the regulation of chromatin condensation, histone acetylation and gene expression. *The EMBO journal* **19**, 6131-6140.

Pile, L. A., Schlag, E. M. and Wassarman, D. A. (2002). The SIN3/RPD3 deacetylase complex is essential for G(2) phase cell cycle progression and regulation of SMRTER corepressor levels. *Molecular and cellular biology* **22**, 4965-4976.

Poortinga, G., Watanabe, M. and Parkhurst, S. M. (1998). Drosophila CtBP: a Hairy-interacting protein required for embryonic segmentation and hairy-mediated transcriptional repression. *The EMBO journal* **17**, 2067-2078.

Ptashne, M. and Gann, A. (1997). Transcriptional activation by recruitment. *Nature* **386**, 569-577.

Pyrowolakis, G., Hartmann, B., Muller, B., Basler, K. and Affolter, M. (2004). A simple molecular complex mediates widespread BMP-induced repression during Drosophila development. *Developmental cell* **7**, 229-240.

Qi, D., Bergman, M., Aihara, H., Nibu, Y. and Mannervik, M. (2008). Drosophila Ebi mediates Snail-dependent transcriptional repression through HDAC3-induced histone deacetylation. *The EMBO journal* **27**, 898-909.

Quinlan, K. G., Nardini, M., Verger, A., Francescato, P., Yaswen, P., Corda, D., Bolognesi, M. and Crossley, M. (2006). Specific recognition of ZNF217 and other zinc finger proteins at a surface groove of C-terminal binding proteins. *Molecular and cellular biology* **26**, 8159-8172.

Radford, S. J., Goley, E., Baxter, K., McMahan, S. and Sekelsky, J. (2005). Drosophila ERCC1 is required for a subset of MEI-9-dependent meiotic crossovers. *Genetics* **170**, 1737-1745.

Ray, R. P. and Schupbach, T. (1996). Intercellular signaling and the polarization of body axes during Drosophila oogenesis. *Genes & development* **10**, 1711-1723.

Reddy, B. A., Bajpe, P. K., Bassett, A., Moshkin, Y. M., Kozhevnikova, E., Bezstarosti, K., Demmers, J. A., Travers, A. A. and Verrijzer, C. P. (2010). Drosophila transcription factor Tramtrack69 binds MEP1 to recruit the chromatin remodeler NuRD. *Molecular and cellular biology* **30**, 5234-5244.

Rinn, J. L., Kertesz, M., Wang, J. K., Squazzo, S. L., Xu, X., Brugmann, S. A., Goodnough, L. H., Helms, J. A., Farnham, P. J., Segal, E. et al. (2007). Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* **129**, 1311-1323.

Rong, Y. S. and Golic, K. G. (2000). Gene targeting by homologous recombination in Drosophila. *Science* **288**, 2013-2018.

Roopra, A., Qazi, R., Schoenike, B., Daley, T. J. and Morrison, J. F. (2004). Localized domains of G9a-mediated histone methylation are required for silencing of neuronal genes. *Molecular cell* **14**, 727-738.
Roose, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., van de Wetering, M., Destree, O. and Clevers, H. (1998). The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature* **395**, 608-612.
Rothstein, R. (1991). Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods in enzymology* **194**, 281-301.
Ruberte, E., Marty, T., Nellen, D., Affolter, M. and Basler, K. (1995). An absolute requirement for both the type II and type I receptors, punt and thick veins, for dpp signaling in vivo. *Cell* **80**, 889-897.
Rubin, G. M. and Spradling, A. C. (1982). Genetic transformation of Drosophila with transposable element vectors. *Science* **218**, 348-353.
Rushlow, C., Colosimo, P. F., Lin, M. C., Xu, M. and Kirov, N. (2001). Transcriptional regulation of the Drosophila gene zen by competing Smad and Brinker inputs. *Genes & development* **15**, 340-351.
Saller, E. and Bienz, M. (2001). Direct competition between Brinker and Drosophila Mad in Dpp target gene transcription. *EMBO reports* **2**, 298-305.
Saller, E., Kelley, A. and Bienz, M. (2002). The transcriptional repressor Brinker antagonizes Wingless signaling. *Genes & development* **16**, 1828-1838.
Sanson, B. (2001). Generating patterns from fields of cells. Examples from Drosophila segmentation. *EMBO reports* **2**, 1083-1088.
Sanyal, A., Lajoie, B. R., Jain, G. and Dekker, J. (2012). The long-range interaction landscape of gene promoters. *Nature* **489**, 109-113.
Schaeper, U., Boyd, J. M., Verma, S., Uhlmann, E., Subramanian, T. and Chinnadurai, G. (1995). Molecular cloning and characterization of a cellular phosphoprotein that interacts with a conserved C-terminal domain of adenovirus E1A involved in negative modulation of oncogenic transformation. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 10467-10471.
Schupbach, T. (1987). Germ line and soma cooperate during oogenesis to establish the dorsoventral pattern of egg shell and embryo in Drosophila melanogaster. *Cell* **49**, 699-707.
Schwank, G. and Basler, K. (2010). Regulation of organ growth by morphogen gradients. *Cold Spring Harb Perspect Biol* **2**, a001669.
Schwartz, Y. B., Kahn, T. G., Nix, D. A., Li, X. Y., Bourgon, R., Biggin, M. and Pirrotta, V. (2006). Genome-wide analysis of Polycomb targets in Drosophila melanogaster. *Nature genetics* **38**, 700-705.
Sekelsky, J. J., Newfeld, S. J., Raftery, L. A., Chartoff, E. H. and Gelbart, W. M. (1995). Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in Drosophila melanogaster. *Genetics* **139**, 1347-1358.
Sharma, V., Swaminathan, A., Bao, R. and Pile, L. A. (2008). Drosophila SIN3 is required at multiple stages of development. *Developmental dynamics : an official publication of the American Association of Anatomists* **237**, 3040-3050.
Shen, J., Dahmann, C. and Pflugfelder, G. O. (2010). Spatial discontinuity of optomotor-blind expression in the Drosophila wing imaginal disc disrupts epithelial architecture and promotes cell sorting. *BMC developmental biology* **10**, 23.

Shi, Y., Sawada, J., Sui, G., Affar el, B., Whetstine, J. R., Lan, F., Ogawa, H., Luke, M. P., Nakatani, Y. and Shi, Y. (2003). Coordinated histone modifications mediated by a CtBP co-repressor complex. *Nature* **422**, 735-738.

Shin, M., Kang, S., Hyun, S. J., Fujita, N., Ishihama, A., Valentin-Hansen, P. and Choy, H. E. (2001). Repression of deoP2 in Escherichia coli by CytR: conversion of a transcription activator into a repressor. *The EMBO journal* **20**, 5392-5399.

Shogren-Knaak, M., Ishii, H., Sun, J. M., Pazin, M. J., Davie, J. R. and Peterson, C. L. (2006). Histone H4-K16 acetylation controls chromatin structure and protein interactions. *Science* **311**, 844-847.

Shravage, B. V., Altmann, G., Technau, M. and Roth, S. (2007). The role of Dpp and its inhibitors during eggshell patterning in Drosophila. *Development* **134**, 2261-2271.

Simon, J. A. and Kingston, R. E. (2009). Mechanisms of polycomb gene silencing: knowns and unknowns. *Nature reviews. Molecular cell biology* **10**, 697-708.

Sipos, L. and Gyurkovics, H. (2005). Long-distance interactions between enhancers and promoters. *The FEBS journal* **272**, 3253-3259.

Sivasankaran, R., Vigano, M. A., Muller, B., Affolter, M. and Basler, K. (2000). Direct transcriptional control of the Dpp target omb by the DNA binding protein Brinker. *The EMBO journal* **19**, 6162-6172.

Small, S., Arnosti, D. N. and Levine, M. (1993). Spacing ensures autonomous expression of different stripe enhancers in the even-skipped promoter. *Development* **119**, 762-772.

Small, S., Blair, A. and Levine, M. (1996). Regulation of two pair-rule stripes by a single enhancer in the Drosophila embryo. *Developmental biology* **175**, 314-324.

Small, S., Kraut, R., Hoey, T., Warrior, R. and Levine, M. (1991). Transcriptional regulation of a pair-rule stripe in Drosophila. *Genes & development* **5**, 827-839.

Song, C. Z., Loewenstein, P. M., Toth, K. and Green, M. (1995). Transcription factor TFIID is a direct functional target of the adenovirus E1A transcription-repression domain. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 10330-10333.

Song, H., Hasson, P., Paroush, Z. and Courey, A. J. (2004). Groucho oligomerization is required for repression in vivo. *Molecular and cellular biology* **24**, 4341-4350.

Spradling, A. C. (1993). Developmental Genetics of Oogenesis. In *The Development of Drosophila melanogaster* (eds M. Bate and A. Martinez Arias). Plainview, N.Y.: Cold Spring Harbor Laboratory Press.

Srinivasan, L. and Atchison, M. L. (2004). YY1 DNA binding and PcG recruitment requires CtBP. *Genes & development* **18**, 2596-2601.

Stadhouders, R., Thongjuea, S., Andrieu-Soler, C., Palstra, R. J., Bryne, J. C., van den Heuvel, A., Stevens, M., de Boer, E., Kockx, C., van der Sloot, A. et al. (2012). Dynamic long-range chromatin interactions control Myb proto-oncogene transcription during erythroid development. *The EMBO journal* **31**, 986-999.

Stanojevic, D., Small, S. and Levine, M. (1991). Regulation of a segmentation stripe by overlapping activators and repressors in the Drosophila embryo. *Science* **254**, 1385-1387.

Stielow, B., Sapetschnig, A., Kruger, I., Kunert, N., Brehm, A., Boutros, M. and Suske, G. (2008). Identification of SUMO-dependent chromatin-associated transcriptional repression components by a genome-wide RNAi screen. *Molecular cell* **29**, 742-754.

Stifani, S., Blaumueller, C. M., Redhead, N. J., Hill, R. E. and Artavanis-Tsakonas, S. (1992). Human homologs of a Drosophila Enhancer of split gene product define a novel family of nuclear proteins. *Nature genetics* **2**, 343.

Stobe, P., Stein, M. A., Habring-Muller, A., Bezdan, D., Fuchs, A. L., Hueber, S. D., Wu, H. and Lohmann, I. (2009). Multifactorial regulation of a hox target gene. *PLoS genetics* **5**, e1000412.
Stokes, D. G., Tartof, K. D. and Perry, R. P. (1996). CHD1 is concentrated in interbands and puffed regions of Drosophila polytene chromosomes. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 7137-7142.
Struffi, P. and Arnosti, D. N. (2005). Functional interaction between the Drosophila knirps short range transcriptional repressor and RPD3 histone deacetylase. *The Journal of biological chemistry* **280**, 40757-40765.
Struffi, P., Corado, M., Kulkarni, M. and Arnosti, D. N. (2004). Quantitative contributions of CtBP-dependent and -independent repression activities of Knirps. *Development* **131**, 2419-2429.
Struhl, G. (1981). A gene product required for correct initiation of segmental determination in Drosophila. *Nature* **293**, 36-41.
Sudou, N., Yamamoto, S., Ogino, H. and Taira, M. (2012). Dynamic in vivo binding of transcription factors to cis-regulatory modules of cer and gsc in the stepwise formation of the Spemann-Mangold organizer. *Development* **139**, 1651-1661.
Sutrias-Grau, M. and Arnosti, D. N. (2004). CtBP contributes quantitatively to Knirps repression activity in an NAD binding-dependent manner. *Molecular and cellular biology* **24**, 5953-5966.
Swaminathan, J., Baxter, E. M. and Corces, V. G. (2005). The role of histone H2Av variant replacement and histone H4 acetylation in the establishment of Drosophila heterochromatin. *Genes & development* **19**, 65-76.
Szuts, D., Freeman, M. and Bienz, M. (1997). Antagonism between EGFR and Wingless signalling in the larval cuticle of Drosophila. *Development* **124**, 3209-3219.
Tautz, D. and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback. *Chromosoma* **98**, 81-85.
Tesfa-Selase, F. and Drabble, W. T. (1996). Specific binding of DnaA protein to a DnaA box in the guaB gene of Escherichia coli K12. *European journal of biochemistry / FEBS* **241**, 411-416.
Thibault, S. T., Singer, M. A., Miyazaki, W. Y., Milash, B., Dompe, N. A., Singh, C. M., Buchholz, R., Demsky, M., Fawcett, R., Francis-Lang, H. L. et al. (2004). A complementary transposon tool kit for Drosophila melanogaster using P and piggyBac. *Nature genetics* **36**, 283-287.
Thomas, K. R., Folger, K. R. and Capecchi, M. R. (1986). High frequency targeting of genes to specific sites in the mammalian genome. *Cell* **44**, 419-428.
Tolhuis, B., Palstra, R. J., Splinter, E., Grosveld, F. and de Laat, W. (2002). Looping and interaction between hypersensitive sites in the active beta-globin locus. *Molecular cell* **10**, 1453-1465.
Tolhuis, B., de Wit, E., Muijrers, I., Teunissen, H., Talhout, W., van Steensel, B. and van Lohuizen, M. (2006). Genome-wide profiling of PRC1 and PRC2 Polycomb chromatin binding in Drosophila melanogaster. *Nature genetics* **38**, 694-699.
Tootle, T. L., Lee, P. S. and Rebay, I. (2003). CRM1-mediated nuclear export and regulated activity of the Receptor Tyrosine Kinase antagonist YAN require specific interactions with MAE. *Development* **130**, 845-857.

Tsai, C. C., Kao, H. Y., Yao, T. P., McKeown, M. and Evans, R. M. (1999). SMRTER, a Drosophila nuclear receptor coregulator, reveals that EcR-mediated repression is critical for development. *Molecular cell* **4**, 175-186.
Tsuda, L., Nagaraj, R., Zipursky, S. L. and Banerjee, U. (2002). An EGFR/Ebi/Sno pathway promotes delta expression by inactivating Su(H)/SMRTER repression during inductive notch signaling. *Cell* **110**, 625-637.
Tsuneizumi, K., Nakayama, T., Kamoshida, Y., Kornberg, T. B., Christian, J. L. and Tabata, T. (1997). Daughters against dpp modulates dpp organizing activity in Drosophila wing development. *Nature* **389**, 627-631.
Turki-Judeh, W. and Courey, A. J. (2012). The unconserved groucho central region is essential for viability and modulates target gene specificity. *PloS one* **7**, e30610.
Turner, J. and Crossley, M. (1998). Cloning and characterization of mCtBP2, a co-repressor that associates with basic Kruppel-like factor and other mammalian transcriptional regulators. *The EMBO journal* **17**, 5129-5140.
Twombly, V., Blackman, R. K., Jin, H., Graff, J. M., Padgett, R. W. and Gelbart, W. M. (1996). The TGF-beta signaling pathway is essential for Drosophila oogenesis. *Development* **122**, 1555-1565.
Um, M., Li, C. and Manley, J. L. (1995). The transcriptional repressor even-skipped interacts directly with TATA-binding protein. *Molecular and cellular biology* **15**, 5007-5016.
Wada, T., Takagi, T., Yamaguchi, Y., Ferdous, A., Imai, T., Hirose, S., Sugimoto, S., Yano, K., Hartzog, G. A., Winston, F. et al. (1998). DSIF, a novel transcription elongation factor that regulates RNA polymerase II processivity, is composed of human Spt4 and Spt5 homologs. *Genes & development* **12**, 343-356.
Wamstad, J. A., Corcoran, C. M., Keating, A. M. and Bardwell, V. J. (2008). Role of the transcriptional corepressor Bcor in embryonic stem cell differentiation and early embryonic development. *PloS one* **3**, e2814.
Wang, L., Charroux, B., Kerridge, S. and Tsai, C. C. (2008). Atrophin recruits HDAC1/2 and G9a to modify histone H3K9 and to determine cell fates. *EMBO reports* **9**, 555-562.
Wang, L., Rajan, H., Pitman, J. L., McKeown, M. and Tsai, C. C. (2006). Histone deacetylase-associating Atrophin proteins are nuclear receptor corepressors. *Genes & development* **20**, 525-530.
Wang, X., Lee, C., Gilmour, D. S. and Gergen, J. P. (2007). Transcription elongation controls cell fate specification in the Drosophila embryo. *Genes & development* **21**, 1031-1036.
Wang, X., Hang, S., Prazak, L. and Gergen, J. P. (2010). NELF potentiates gene transcription in the Drosophila embryo. *PloS one* **5**, e11498.
Wang, Y., Zhang, H., Chen, Y., Sun, Y., Yang, F., Yu, W., Liang, J., Sun, L., Yang, X., Shi, L. et al. (2009). LSD1 is a subunit of the NuRD complex and targets the metastasis programs in breast cancer. *Cell* **138**, 660-672.
Waring, G. L. (2000). Morphogenesis of the eggshell in Drosophila. *Int Rev Cytol* **198**, 67-108.
Wehn, A. and Campbell, G. (2006). Genetic interactions among scribbler, Atrophin and groucho in Drosophila uncover links in transcriptional repression. *Genetics* **173**, 849-861.
Wesolowska, N. and Rong, Y. S. (2013). Long-range targeted manipulation of the Drosophila genome by site-specific integration and recombinational resolution. *Genetics* **193**, 411-419.
Williams, D. R., Motallebi-Veshareh, M. and Thomas, C. M. (1993). Multifunctional repressor KorB can block transcription by preventing isomerization of RNA polymerase-promoter complexes. *Nucleic acids research* **21**, 1141-1148.

Winkler, C. J., Ponce, A. and Courey, A. J. (2010). Groucho-mediated repression may result from a histone deacetylase-dependent increase in nucleosome density. *PloS one* **5**, e10166.
Winter, S. E. and Campbell, G. (2004). Repression of Dpp targets in the Drosophila wing by Brinker. *Development* **131**, 6071-6081.
Wollmann, P., Cui, S., Viswanathan, R., Berninghausen, O., Wells, M. N., Moldt, M., Witte, G., Butryn, A., Wendler, P., Beckmann, R. et al. (2011). Structure and mechanism of the Swi2/Snf2 remodeller Mot1 in complex with its substrate TBP. *Nature* **475**, 403-407.
Wolpert, L. (1969). Positional information and the spatial pattern of cellular differentiation. *J Theor Biol* **25**, 1-47.
Wu, X., Tanwar, P. S. and Raftery, L. A. (2008). Drosophila follicle cells: morphogenesis in an eggshell. *Semin Cell Dev Biol* **19**, 271-282.
Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. *Development* **117**, 1223-1237.
Xu, X., Yin, Z., Hudson, J. B., Ferguson, E. L. and Frasch, M. (1998). Smad proteins act in combination with synergistic and antagonistic regulators to target Dpp responses to the Drosophila mesoderm. *Genes & development* **12**, 2354-2370.
Yakoby, N., Lembong, J., Schupbach, T. and Shvartsman, S. Y. (2008). Drosophila eggshell is patterned by sequential action of feedforward and feedback loops. *Development* **135**, 343-351.
Yamaguchi, Y., Takagi, T., Wada, T., Yano, K., Furuya, A., Sugimoto, S., Hasegawa, J. and Handa, H. (1999). NELF, a multisubunit complex containing RD, cooperates with DSIF to repress RNA polymerase II elongation. *Cell* **97**, 41-51.
Yeung, K., Kim, S. and Reinberg, D. (1997). Functional dissection of a human Dr1-DRAP1 repressor complex. *Molecular and cellular biology* **17**, 36-45.
You, A., Tong, J. K., Grozinger, C. M. and Schreiber, S. L. (2001). CoREST is an integral component of the CoREST- human histone deacetylase complex. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 1454-1458.
Yudkovsky, N., Ranish, J. A. and Hahn, S. (2000). A transcription reinitiation intermediate that is stabilized by activator. *Nature* **408**, 225-229.
Zeitlinger, J., Stark, A., Kellis, M., Hong, J. W., Nechaev, S., Adelman, K., Levine, M. and Young, R. A. (2007). RNA polymerase stalling at developmental control genes in the Drosophila melanogaster embryo. *Nature genetics* **39**, 1512-1516.
Zhang, C. L., McKinsey, T. A., Lu, J. R. and Olson, E. N. (2001). Association of COOH-terminal-binding protein (CtBP) and MEF2-interacting transcription repressor (MITR) contributes to transcriptional repression of the MEF2 transcription factor. *The Journal of biological chemistry* **276**, 35-39.
Zhang, G., Campbell, E. A., Minakhin, L., Richter, C., Severinov, K. and Darst, S. A. (1999). Crystal structure of Thermus aquaticus core RNA polymerase at 3.3 A resolution. *Cell* **98**, 811-824.
Zhang, H. and Levine, M. (1999). Groucho and dCtBP mediate separate pathways of transcriptional repression in the Drosophila embryo. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 535-540.
Zhang, H., Levine, M. and Ashe, H. L. (2001). Brinker is a sequence-specific transcriptional repressor in the Drosophila embryo. *Genes & development* **15**, 261-266.
Zhang, Q., Piston, D. W. and Goodman, R. H. (2002). Regulation of corepressor function by nuclear NADH. *Science* **295**, 1895-1897.

Zhang, Q., Wang, S. Y., Fleuriel, C., Leprince, D., Rocheleau, J. V., Piston, D. W. and Goodman, R. H. (2007). Metabolic regulation of SIRT1 transcription via a HIC1:CtBP corepressor complex. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 829-833.
Zhang, S., Xu, L., Lee, J. and Xu, T. (2002). Drosophila atrophin homolog functions as a transcriptional corepressor in multiple developmental processes. *Cell* **108**, 45-56.
Zhao, L. J., Subramanian, T., Zhou, Y. and Chinnadurai, G. (2006). Acetylation by p300 regulates nuclear localization and function of the transcriptional corepressor CtBP2. *The Journal of biological chemistry* **281**, 4183-4189.
Zhou, W., Huang, J., Watson, A. M. and Hong, Y. (2012). W::Neo: a novel dual-selection marker for high efficiency gene targeting in Drosophila. *PloS one* **7**, e31997.
Zhu, X., Ahmad, S. M., Aboukhalil, A., Busser, B. W., Kim, Y., Tansey, T. R., Haimovich, A., Jeffries, N., Bulyk, M. L. and Michelson, A. M. (2012). Differential regulation of mesodermal gene expression by Drosophila cell type-specific Forkhead transcription factors. *Development* **139**, 1457-1466.